SYNTHESES, CHARACTERISATION, CATALYTIC ACTIVITY AND BIOLOGICAL PROPERTY OF NICKEL(II) COMPLEXES OF DESIGNED MANNICH BASE LIGANDS
Abstract

With an aim to study the electronic effect of the group lying at para-position of phenol-based compartmental Mannich-base ligands, five dinuclear nickel(II) complexes \([\text{Ni}_2L(1-5)(\mu-\text{NO}_3)(\text{NO}_3)_2]\) have been synthesized with variation in the para-substitution (R= Me, CHMe₂, CMe₃, Cl, OMe) leading to closely similar structures in each case. They have been characterized by FTIR, UV-vis, ESI-MS, TGA and cyclic voltammetry. Molecular structures of four (1-4) out of five complexes have been confirmed by single-crystal X-ray diffraction. The phosphatase activity has been studied with the phosphomonoester 4-nitrophenylphosphate (4-NPP) in DMSO-water medium (3:1) at pH 9 where the increased concentration of the end product 4-nitriphenolate with time has been monitored by UV-Vis spectrophotometry leading to \(k_{cat}\) values in the range 6-81 sec\(^{-1}\). The catechol oxidase activity has been investigated in DMSO-methanol with the model substrate 3,5-DTBC in completely aerobic conditions where they exhibit moderate catecholase activity having \(k_{cat}\) values in the range \((4-11)\times10^{-4}\) sec\(^{-1}\). Moreover the cytotoxic potential of 1-5 along with the mechanism have been explored. On the basis of \(k_{cat}\) values and bioactivity it is evident that complex-3 has the highest catalytic efficiency.

Keywords: Mannich-base; Ni\(^{II}\); Phosphatase; Catecholase; Apoptosis.
6A.1. Introduction

Cancer is undoubtedly one of the main health concerns facing our society and one of the primary targets regarding medicinal chemistry.\textsuperscript{1} Even though platinum based complexes had been in primary focus of research on chemotherapy agents,\textsuperscript{2-4} the interests in this field have shifted to non platinum based agents, in order to find different metal complexes with less side effects and similar, or better, cytotoxicity.\textsuperscript{5-7} Thus, a wide variety of metal complexes based on titanium, gallium, palladium, gold, cobalt, ruthenium and tin are being intensively studied as platinum replacements.\textsuperscript{5-7} Furthermore, Nickel (II) based complexes appear to be very promising candidates for anticancer therapy; an idea supported by a considerable number of research articles describing the synthesis and cytotoxic activities of numerous nickel (II) complexes.\textsuperscript{8} However, nickel (II) complexes have potential application in medicine. A large number of these compounds have been extensively studied because of their antibacterial, fungicidal and anticancer activities.\textsuperscript{8,9} The complexes of Ni\textsuperscript{II} showed high activity and selectivity against human cancer cell lines.\textsuperscript{8} Biological studies, performed with these complexes, including inhibition of cell proliferation and apoptosis test \textit{in vitro} on the human leukemia cell line, indicated that some of them could induce apoptosis.\textsuperscript{8,9} The present study was to evaluate the cytotoxic potential of different nickel (II) complex with exploration of mechanism involved.

6A.2. Experimental section

\textbf{Physical methods and materials.} The \textit{p}-substituted phenols were obtained from different commercial sources as were available to us namely Merck (\textit{p}-cresol), Sigma-Aldrich (4-isopropylphenol, 4-\textit{t}-butylphenol and 4-methoxyphenol) and Loba chemic (4-chlorophenol). Nickel nitrate hexahydrate was bought from Merck. 3,5-di-\textit{t}-butylcatechol (3,5-DTBC) and (4-nitrophenyl)phosphate (4-NPP) was purchased from Sigma-Aldrich and Spectrochem respectively. 4-NPP was recrystallized from ethanol/water before use. Reaction solutions for DTBC oxidation and 4-NPP cleavage
were prepared according to the standard sterile techniques. UV-Visible spectra and kinetic traces were monitored with a Schimadzu 2450 UV/Vis spectrophotometer equipped with multiple cell-holders and thermostat. 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, antibiotics (penicillin, streptomycin) and trypsin-EDTA solution were obtained from Gibco BRL-Life Technologies (Gibco, NY). All other reagents, kits and solvents used were procured from Merck Chemicals (Germany).

**Syntheses of Ligands.**

*Synthesis of HL1.* To an ethanolic solution (30 ml) of \( p \)-cresol (30 mmol, 3.24 gms), 2-(2-Methylaminoethyl)pyridine (90 mmol, 4.09 gms) was added dropwise with constant stirring. After 30 minutes, 37% (w/v) formalin solution (90 mmol, 7.5 ml) was added to it. The resulting mixture was stirred for an additional one hour at room temperature and then refluxed for 24 hours. It was evaporated under reduced pressure and the yellow oil was extracted with saturated brine solution and dichloromethane several times. The organic phase was separated, dried with anhydrous MgSO\(_4\), concentrated by evaporation of ether and subsequently vacuum dried for the removal of last traces of water. The liquid ligand was further purified by flash column chromatography (silica gel, hexane : ethylacetate : triethylamine = 100 : 5 : 1) to afford the desired \( \beta \)-aminophenol.

Yield=5.72 gm (47%). Elemental analysis calcd (%) for C\(_{25}\)H\(_{31}\)N\(_4\)O\(_1\) : C 74.41, H 7.74, N 13.88, O 3.97; found: C 74.93, H 7.88, N 13.54, O 3.65. IR data (NaCl plate): \( \gamma_{\text{bar}} \) = 1257, 1435, 1475, 1569, 1592 cm\(^{-1}\);

*Synthesis of HL2.* The same procedure was followed as HL1 where 4-isopropyl-phenol (30 mmol, 4.09 gms) was used instead of \( p \)-cresol. Elemental analysis calcd (%) for C\(_{25}\)H\(_{35}\)N\(_4\)O\(_1\) : C 74.41, H 7.74, N 13.88, O 3.97; found: C 74.93, H 7.88, N 13.54, O 3.65.

Yield=6.64 gm (51%). Elemental analysis calcd (%) for C\(_{25}\)H\(_{35}\)N\(_4\)O\(_1\) : C 75.14, H 8.17, N 12.98, O 3.71; found: C 75.48, H 8.25, N 12.66, O 3.61. IR data (NaCl plate): \( \gamma_{\text{bar}} \) = 1123, 1250, 1435, 1475, 1591 cm\(^{-1}\);
Synthesis of HL3. This ligand was prepared by identical process as HL1 with 4-tert-butyl-phenol (30 mmol, 4.51 gms) as the starting material instead of p-cresol.

Yield=6.18 gm (46%). Elemental analysis calcd (%) for C$_{28}$H$_{37}$N$_{4}$O$_{1}$: C 75.47, H 8.37, N 12.57, O 3.59; found: C 75.58, H 8.49, N 12.45, O 3.48. IR data (NaCl plate): $\gamma_{\text{bar}}$= 1255, 1503, 1435, 1475, 1569, 1591 cm$^{-1}$;

Synthesis of HL4. A similar procedure was adopted here wherein 4-chlorophenol (30 mmol, 3.86 gmns) was the active phenolic compound.

Yield=7.04 gm (55%). Elemental analysis calcd (%) for C$_{28}$H$_{28}$N$_{4}$Cl$_{1}$O$_{1}$: C 67.99, H 6.66, N 13.21, O 3.77, Cl 8.37; found: C 68.31, H 6.75, N 13.93, O 3.68, Cl 8.33. IR data (NaCl plate): $\gamma_{\text{bar}}$= 1218, 1436, 1473, 1569, 1591 cm$^{-1}$;

Synthesis of HL5. It was synthesized by the same process as above with 4-methoxyphenol (30 mmol, 3.86 gmns) as the starting compound.

Yield=6.58 gm (52%). Elemental analysis calcd (%) for C$_{25}$H$_{31}$N$_{4}$O$_{2}$: C 71.57, H 7.45, N 13.35, O 7.63; found: C 71.63, H 7.59, N 13.28, O 7.50. IR data (NaCl plate): $\gamma_{\text{bar}}$= 1149, 1437, 1472, 1495, 1593, 1655 cm$^{-1}$;

Syntheses of complexes.

Synthesis of complex-1. To a methanolic solution (10 ml) of HL1 (0.5 mmol, 203 mg), a methanolic solution (10 ml) of Ni(NO$_{3}$)$_{2}$.6H$_{2}$O (1 mmol, 291 mg) was added. The light yellow reaction mixture was stirred for 30 mins. After 2 days, suitable X-ray quality green-coloured square-shaped single crystals were obtained.

Yield=286 mg (81%). Elemental analysis calcd (%) for C$_{25}$H$_{31}$N$_{4}$Ni$_{2}$O$_{10}$: C 42.47, H 4.42, N 13.87, Ni 16.61, O 22.63; found C 42.48, H 4.40, N 13.88, Ni 16.63 O 22.61. IR data (NaCl plate): $\gamma_{\text{bar}}$= 766, 1021, 1288, 1384, 1479, 1509, 1610, 1634 cm$^{-1}$;

Synthesis of complex-2. The same procedure was adopted where HL2 (0.5 mmol, 217 mg) was used instead of HL1 and good crystallographic quality rhombic green coloured single crystals were obtained.
Yield = 30.48 mg (83%). Elemental analysis calcd (%) for C\textsubscript{27}H\textsubscript{36.6}N\textsubscript{7}Ni\textsubscript{2}O\textsubscript{10.8}: C 43.27, H 4.92, N 13.08, Ni 15.67, O 23.06; found C 43.28, H 4.94, N 13.06, Ni 15.65 O 23.07. IR data (NaCl plate): γ\textsubscript{bar}= 771, 1023, 1301, 1478, 1609, 2968 cm\textsuperscript{-1};

*Synthesis of complex-3.* The same procedure was adopted where HL3 (0.5 mmol, 224 mg) was used instead of HL1 and needle-shaped green coloured single crystals were obtained for X-ray diffraction.

Yield = 28.81 mg (77%). Elemental analysis calcd (%) for C\textsubscript{28}H\textsubscript{37}N\textsubscript{7}Ni\textsubscript{2}O\textsubscript{10}: C 44.90, H 4.98, N 13.09, Ni 15.67, O 21.36; found C 44.89, H 4.98, N 13.10, Ni 15.66 O 21.37. IR data (NaCl plate): γ\textsubscript{bar}= 967, 1026, 1306, 1384, 1449, 1611 cm\textsuperscript{-1};

*Synthesis of complex-4.* The same procedure was adopted where HL4 (0.5 mmol, 213 mg) was used instead of HL1 to yield square-shaped green single crystals.

Yield = 25.80 mg (71%). Elemental analysis calcd (%) for C\textsubscript{24}H\textsubscript{29.4}ClN\textsubscript{7}Ni\textsubscript{2}O\textsubscript{10.7}: C 38.96, H 4.00, Cl 4.79, N 13.25, Ni 15.87, O 23.13; found C 38.97, H 4.01, Cl 4.77, N 13.26, Ni 15.88 O 23.11. IR data (NaCl plate): γ\textsubscript{bar}= 772, 968, 1022, 1291, 1469, 14991610, 1632, 3368 cm\textsuperscript{-1};

*Synthesis of complex-5.* The same procedure was adopted where HL5 (0.5 mmol, 211 mg) was used instead of HL1 and a green coloured compound was obtained.

Yield = 31.78 mg (88%). Elemental analysis calcd (%) for C\textsubscript{25}H\textsubscript{31}N\textsubscript{7}Ni\textsubscript{2}O\textsubscript{11}: C 41.53, H 4.32, N 13.56, Ni 16.25, O 24.34; found C 41.54, H 4.33, N 13.55, Ni 16.26 O 24.32. IR data (NaCl plate): γ\textsubscript{bar}= 797, 1024, 1159, 1288, 1446, 1480, 1609, 2933, 3401 cm\textsuperscript{-1};

*X-ray crystal structure analysis.* The X-ray single-crystal data of compounds 1-4 were collected on a Bruker-AXS APEX CCD diffractometer at 296(2) K. The crystallographic data, conditions retained for the intensity data collection and some features of the structure refinements are listed in Table 6A.1. The intensities were collected with Mo-Kα radiation (λ = 0.71073 Å). Data processing, Lorentz-polarization and absorption corrections were performed using APEX, SAINT and the SADABS computer programs. The structures were solved by direct methods and refined by full-matrix least-squares methods on F\textsuperscript{2}, using the SHELXTL. 

218
program package. All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were located from difference Fourier maps, assigned with isotropic displacement factors and included in the final refinement cycles by use of HFIX (parent C atom) or DFIX (parent O atom) utility of the SHELXTL program. Molecular plots were performed with the Mercury program. In case of 2 and 4 occupancy factors of 0.80 and 0.70 were applied to lattice water molecules, respectively.

**Table 6A.1. Crystallographic data and processing parameters for 1 – 4.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>1</th>
<th>2 0.8(H₂O)</th>
<th>3</th>
<th>4 0.7(H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical formula</td>
<td>C₂₅H₃₁N₇Ni₂O₁₀</td>
<td>C₂₇H₃₆.₆N₇Ni₂O₁₀.₈</td>
<td>C₂₈H₃₇N₇Ni₂O₁₀</td>
<td>C₂₄H₂₉.₄ClN₇Ni₂O₁₀.₇</td>
</tr>
<tr>
<td>Formula mass</td>
<td>706.95</td>
<td>749.41</td>
<td>749.03</td>
<td>739.98</td>
</tr>
<tr>
<td>System</td>
<td>Monoclinic</td>
<td>Monoclinic</td>
<td>Monoclinic</td>
<td>Monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P₂₁/n</td>
<td>P₂₁/n</td>
<td>Cc</td>
<td>P₂₁/n</td>
</tr>
<tr>
<td>a (Å)</td>
<td>10.6333(13)</td>
<td>10.6724(16)</td>
<td>14.1480(5)</td>
<td>10.6743(11)</td>
</tr>
<tr>
<td>b (Å)</td>
<td>15.630(2)</td>
<td>16.421(2)</td>
<td>15.3628(5)</td>
<td>15.6272(17)</td>
</tr>
<tr>
<td>c (Å)</td>
<td>18.033(2)</td>
<td>18.430(3)</td>
<td>17.1155(6)</td>
<td>18.068(2)</td>
</tr>
<tr>
<td>α (°)</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>β (°)</td>
<td>97.823(2)</td>
<td>95.785(2)</td>
<td>111.275(1)</td>
<td>97.609(3)</td>
</tr>
<tr>
<td>γ (°)</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>V (Å³)</td>
<td>2969.2(6)</td>
<td>3213.3(8)</td>
<td>3466.6(2)</td>
<td>2087.4(6)</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>μ (mm⁻¹)</td>
<td>1.335</td>
<td>1.420</td>
<td>1.148</td>
<td>1.419</td>
</tr>
</tbody>
</table>
### Phosphatase activity

To study the phosphatase activity of the Ni complexes, disodium salt of (4-nitrophenyl)phosphate hexahydrate was the preferred choice as the substrate. Their hydrolytic tendency was detected spectrophotometrically by monitoring the time evolution of *p*-nitrophenolate (λ<sub>max</sub>=420 nm) through wavelength scan from 200-800 nm in DMSO-buffer (3:1) at pH 9 where substrate was in 20 equivalents of the catalyst, till roughly 2% reaction conversion.<sup>13</sup> The kinetic study of complex-1-5 was done by the initial slope method following the rate of increase in absorption of the band at ~ 420 nm corresponding to rise in 4-nitrophenolate concentration. The details are described in Chapter 2A.

### Computational Details

Gaussian 09 program package was utilized to perform the calculations. B3LYP/6-31G(d) (LANL2DZ with Los Alamos ECPs for Ni) functional-basis-set combination was adopted in geometry optimization and frequency analysis due to

<table>
<thead>
<tr>
<th>Dcalc (Mg/m&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>1.582</th>
<th>1.549</th>
<th>1.435</th>
<th>1.645</th>
</tr>
</thead>
<tbody>
<tr>
<td>θ&lt;sub&gt;max&lt;/sub&gt; (°)</td>
<td>25.30</td>
<td>25.30</td>
<td>33.00</td>
<td>27.00</td>
</tr>
<tr>
<td>Data collected</td>
<td>20601</td>
<td>22065</td>
<td>30628</td>
<td>36623</td>
</tr>
<tr>
<td>Unique refl. / Rint</td>
<td>5383 / 0.0603</td>
<td>5826 / 0.0249</td>
<td>10346 / 0.0259</td>
<td>6426 / 0.0279</td>
</tr>
<tr>
<td>Parameters / Restraints</td>
<td>400 / 0</td>
<td>434 / 2</td>
<td>429 / 2</td>
<td>414 / 2</td>
</tr>
<tr>
<td>Goodness-of-Fit on F2</td>
<td>1.052</td>
<td>1.027</td>
<td>1.046</td>
<td>1.048</td>
</tr>
<tr>
<td>R1</td>
<td>0.0443</td>
<td>0.0331</td>
<td>0.0398</td>
<td>0.0352</td>
</tr>
<tr>
<td>wR2 (all data)</td>
<td>0.1316</td>
<td>0.0940</td>
<td>0.1117</td>
<td>0.1044</td>
</tr>
</tbody>
</table>
its relative cheap computational expense and maintaining satisfactory data accuracy. Single point energies with empirical Grimme’s dispersion with Becke-Johnson damping GD3BJ corrections in liquid phase were refined using B3LYP/6-311++G(d,p) (Stuttgart/Dresden SDD with ECPs for Ni) combination. Supermolecule simulation in implicit solvent mode (solvent = DMSO, Eps = 46.826) using single point polarizable continuum model (PCM) with the Truhlar and coworkers’ SMD radii and non-electrostatic terms, was employed to rationalize the role of solvent participation during reaction and to obtain energies in liquid phase. Frequency analysis and intrinsic reaction coordinate (IRC) calculation with HPC algorithm were utilized to distinguish transition states from local minima and to verify their connections. Natural bond orbital (NBO) was performed on the optimized structures for population analysis. All of the thermodynamic data were obtained at 298.15 K.

**Catechol Oxidase Activity.** The catalytic activity of the complexes were screened by treating $1 \times 10^{-4}$ (M) complex solutions with $1 \times 10^{-2}$ (M) of 3,5-DTBC in DMSO-methanol medium. The details are described in Chapter 2B.14

**Cell culture.** HepG2 (Human hepatocellular carcinoma) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (PSN) at 37°C in a humidified atmosphere under 5% CO$_2$. After 75-80% confluency, cells were harvested with 0.025% trypsin and 0.52 mM EDTA in phosphate buffered saline (PBS), and seeded at desired density to allow them to re-equilibrate a day before the start of experiment.

**Determination of cell viability using MTT assay.** The capacity of nickel (II) complex to interfere with the growth of HepG2 cells was determined with the aid of MTT assay.15 Briefly, HepG2 cells with a density $1 \times 10^6$ cells per well were pre cultured into 96 well micro titer plates for 8 hr, 16 hr, 24 hr and 48 hr at 37°C, 5% CO$_2$. Complexes 1, 2, 3, 4 and 5 were added in micro wells containing the cell culture at final concentrations of 0–80 μM. Then each well was loaded with 10 μl of MTT solution (5 mg mL$^{-1}$ in PBS pH=7.4) for 4 hr at 37°C. The insoluble formazan was dissolved in 100 μl of DMSO, and the cell viability was determined by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader (Bio Rad, USA). All experiments were performed
in triplicate, and the percentage of cell viability was calculated according to the following equation:

$$\text{Cell viability (\%)} = \frac{A_{570} \text{ (Sample)}}{A_{570} \text{ (Control)}} \times 100 \%$$

Where $A_{570}$ (sample) refers to the reading from the wells treated with nickel (II) complex and $A_{570}$ (control) refers to that from the wells treated with medium containing 10% PBS only.

**Measurement of lactate dehydrogenase release.** LDH activity was determined by the linear region of a pyruvate standard graph using regression analysis. Briefly, 1 ml of buffered substrate (lithium lactate) and 0.1 ml of the cell supernatant media were added and tubes were incubated at 37°C for 30 min. After adding 200 μl of NAD solution, the incubation was continued for another 30 min. The reaction was then arrested by adding 100 μl of DNPH reagent and the solution were incubated for a further period of 15 min at 37°C. After this 100 μl of cell supernatant media were added to blank tubes after arresting the reaction with DNPH. A 3.5-ml aliquot of 0.4 N sodium hydroxide was added to all the tubes. The colour developed was measured at 420 nm in a UV/visible spectrophotometer (Bio Rad, USA).

**Determination of cellular nitrate.** Cellular nitrite formation was determined using Griess reagent. 990 μl of Griess reagent was mixed with 10 μl of the cell lysate. After 5 minute of incubation at 37°C in the dark, the absorbance was taken at 540 nm using spectrophotometer and the value was calculated from standard curve using 1 mM NaNO₂ solution.

**Determination of lipid peroxidation.** Thiobarbituric acid reactive substance (TBARS) in the cell lysate was estimated using standard protocol. Briefly, the homogenate was incubated with 15% TCA, 0.375% TBA and 5 N HCl at 95°C for 15 minutes; then the mixture was cooled and centrifuged. The absorbance of the supernatant was measured at 535 nm against appropriate blank. Lipid peroxidation was expressed as amount of produced TBARS in nmol/mg protein.
Quantification of apoptosis and necrosis using Annexin-V FITC/PI. Apoptosis was assayed by Annexin-V FITC/PI apoptosis detection kit (Calbiochem, CA, USA). Briefly, cells were treated with or without nickel (II) complex-3, then washed and stained with Annexin-V-FITC (10 μg/ml) and PI (20 μg/ml) and incubated 37°C for 15 min in dark accordance with the manufacturer’s instructions. The percentages of live, apoptotic and necrotic cells were determined by flowcytometric method using excitation/emission wavelengths of 488/525 nm and 488/675 nm for Annexin-V and PI, respectively (Beckton Dickinson, San Jose, CA, USA).19

Immunofluorescence study. Cells were fixed with 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100. Blocking solution (2% BSA and 0.1% Triton X-100 in PBS) was then added for 1 hr before the slides were incubated with anti γH2AX, anti-p53 and anti-Bcl2 antibodies (1:250 each in blocking solution), followed by incubation with Rhodamine and FITC-labeled anti-mouse and anti-rabbit antibodies (1:250 each in blocking solution). The slides were washed, covered with mounting solution and visualized using Olympus confocal microscope.20

Western blot analysis. After HepG2 cells were co-cultured with 40 μM of nickel (II) complex-3 for 8 hr, 16 hr and 24 hr, cells were washed with cold PBS and subsequently lysed in ice-cold lysis buffer consisting of 50 mmol/L Tris–HCl (pH-8.0), 50 mmol/L KCl, 5 mmol/L DTT, 1 mmol/L EDTA, 0.1% SDS, 0.5% Triton X-100 and protease inhibitor cocktail tablets (Roche, Indianapolis, Indiana) for 5 min on ice. Protein concentration was determined with the Lowry protein assay method. Total protein was separated by 12.5 % SDS polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica). After blocking with 5% non-fat milk, the membranes were incubated successively with a primary anti-mouse antibody against Bcl2, Bax (Santa Cruz, CA) and β-actin (Cell signaling Technology, MA) overnight at 4°C. The membrane was rinsed and washed 3 times with TBS containing 0.1% Tween 20 and probed with the appropriate alkaline phosphatase conjugated secondary antibody for 2 h at room temperature. The proteins were then visualized following incubation with NBT-BCIP solution.21
Measurement of caspase 3 and caspase 9 activity. Levels of pro caspases were assayed by commercially available colorimetric assay kits (Bio Vision Research Products, Mountain View, CA) in accordance with the manufacturer’s instructions.\textsuperscript{22}

Measurement of mitochondrial transmembrane potential. Mitochondrial dysfunction was determined by measuring changes in the mitochondrial membrane potential (MMP, $\Delta \Psi m$) using flow cytometry after staining live cells with JC-1 ($5,5',6,6'$-tetrachloro-1,1',3,3'-tetrethylbenzimidalylcarbocyanine iodide).\textsuperscript{23} Briefly, cells were resuspended at $1 \times 10^6$/mL in pre-warmed JC-1 working buffer containing 5 μg/mL JC-1 and incubated for 15 min at 37°C in 5% CO₂. Subsequently, the cells were washed in assay buffer and directly analyzed in a BD FACSAlert III cytometer (Becton Dickinson, Heidelberg, Germany). Photomultiplier settings were adjusted to detect JC-1 monomer fluorescence signals on the FL1 detector (green fluorescence, centered around 525 nm) and JC-1 aggregate fluorescence signals on the FL2 detector (red fluorescence, centered around 590 nm). The relative monomer (green) fluorescence intensity values were used for data presentation.

Measurement of ROS generation. ROS were measured with the non-fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA).\textsuperscript{24} DCFH-DA passively diffuses into cells and is hydrolyzed by cellular esterase’s to 2',7'-dichlorofluorescin (DCFH), a non-fluorescent molecule that can be oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of peroxides. Briefly, HepG2 cells were seeded into 24-well plates and co-incubated with nickel (II) complex-3 (40 μM) for 8 hr, 16 hr and 24 hr. Then cells were incubated with 10 mM DCFH-DA which dissolved in cell-free medium at 37°C for 30 min and then washed three times with PBS. Cellular fluorescence was analysed using flowcytometer (Beckton Dickinson, San Jose, CA, USA) at an excitation of 488 nm and an emission of 525 nm.

Measurement of intracellular free Ca$^{2+}$. The free cytosolic Ca$^{2+}$ concentration was determined using the fluorescent calcium indicator Fura 2-acetoxymethy ester (Fura2-AM). After HepG2 cells were treated with nickel (II) complex-3 (40 μM) for 8 hr, 16 hr and 24 hr and preloaded with 1 μM Fura2-AM for 30 min in the dark at 37°C in a
humidified incubator. After loading, the cells were collected and gently rinsed with D-Hanks solution, then resuspended in D-Hanks solution containing 0.2% BSA at the concentration of 10^6 cells/ml. Intracellular free Ca^{2+} was measured at emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm on fluorescence plate reader (Perkin Elmer, Massachusetts, USA). The ratio of fluorescence intensity at 340–380 nm (F340/F380) was used to estimate intracellular free calcium.  

**Determination of reduced glutathione activity.** Cell lysate was treated with 0.1 ml of 25% TCA, and the resulting precipitate was pelleted by centrifugation at 3,900×g for 10 minutes. The free endogenous sulfhydryl was assayed in a 3 mL volume mixture (2 mL of 0.5 mM DTNB prepared in 0.2 M phosphate buffer, with 1 mL of cell supernatant). The GSH when reacts with DTNB forms a yellow complex with DTNB, whose absorbance was read at 412 nm.  

**Determination of superoxide dismutase activity.** Superoxide dismutase activity was determined using the slightly modified pyrogallol autoxidation method. Briefly, cell lysate was added to 62.5 mM tris cacodylic acid buffer followed by the addition of 4 mM pyrogallol. The autoxidation of pyrogallol was monitored at 420 nm.  

**Determination of catalase activity.** Cell lysate (containing 50 μg of protein) was added to 50 mM phosphate buffer (pH-7) containing 100 mM \( \text{H}_2\text{O}_2 \) and incubated for 2 min at 37°C. The absorbance was monitored at 240 nm for 5 min. The change in absorbance with time was proportional to the breakdown of \( \text{H}_2\text{O}_2 \). The Catalase activity was expressed as units/mg protein and one unit of enzyme activity was defined as the amount of enzyme required to breakdown 1 μM \( \text{H}_2\text{O}_2 \).  

**Statistical analysis.** All data are presented as the mean ± SEM. The significance of differences between treated and untreated groups were determined by One-Way ANOVA test using OriginLab 8.0 software. p<0.05 value was considered as significant.
6A.3. Results and Discussion

Syntheses of compounds. The ligands were prepared by standard Mannich reaction\textsuperscript{13} by using an activated phenolic compound, a suitable secondary amine, 2-(2-Methylaminoethyl)pyridine in our case and formalin. Though the appropriate conditions give the target molecule in major percentage, column chromatographic separation is essential to isolate it from a mixture of minor amount of one-sided product and unreacted amine. Five different ligands have been prepared by slightly varying the para-substitution with the sole aim of studying the electronic effect of various functional groups (methyl, isopropyl, tertiary, chloro and methoxy) on the catalytic activities of interest. The ligand HL\textsubscript{1} is a reported ligand\textsuperscript{29} while the other four are new though there is sufficient parity among all of them. They have been characterized by FT-IR, thermogravimetric analyses, elemental analyses, UV-vis spectra (Figure 6A.1-6A.3), ESI-MS analyses and single-crystal XRD. The IR and TGA spectra did not contain any major characteristic signals and so have not been added here.

![Chemical structure of ligand (HL1-5) and complex (complex-1-5)](image)

*Scheme 6A.1.* Chemical structure of ligand (HL\textsubscript{1-5}) and complex (complex-1-5) where \(R= \text{CH}_3\) (HL\textsubscript{1}, complex-1), CHMe\textsubscript{2} (HL\textsubscript{2}, complex-2), CMe\textsubscript{3} (HL\textsubscript{3}, complex-3), Cl (HL\textsubscript{4}, complex-4), OCH\textsubscript{3} (HL\textsubscript{5}, complex-5).
Electronic spectra. The solid Ni\textsuperscript{II} complexes show three spin-allowed d–d transitions around 1,200 ($^3A_{2g} \rightarrow ^3T_{2g}$), 650 ($^3A_{2g} \rightarrow ^3T_{1g}(F)$) and 450 ($^3A_{2g} \rightarrow ^3T_{1g}(P)$) nm along with an LMCT band which remains intact in solution proving a distorted octahedral geometry in both phase (Figure 6A.17-6A.19).

Figure 6A.1. Solid state electronic spectra of the complex-1-5 at 298 K.

Figure 6A.2. Electronic spectra of complexes in DMSO of (A) $5 \times 10^{-5}$ (M). Inset shows the same for $1 \times 10^{-3}$ (M) solution. (B) $5 \times 10^{-3}$ (M) solution at 298 K.
Figure 6A.3. Electronic spectra of complexes in 75% DMSO at pH 9 of (A) $5 \times 10^{-5}$(M) solution and (B) $5 \times 10^{-3}$(M) at 298 K.

Description of the crystal structures. The crystal structures of 1 - 4 consists of neutral dinuclear complex units $[\text{Ni}_2\text{L}(\text{1-4})(\text{NO}_3)_3]$, ($\text{L} = \text{Mannich-base ligand, para-substituted R}=\text{Me, CHMe}_2, \text{CMe}_3, \text{Cl}$). Crystal structures of 2 and 4 co-crystallize with 0.80 and 0.70 lattice water molecules per dinuclear unit. Perspective views of the dinuclear units together with partial atom numbering schemes are given in Figures 6A.4 – 6A.5, respectively. Each Ni$^{II}$ center within a dinuclear complex unit is octahedrally coordinated by two N donor atoms and the deprotonated O(1) atom of the phenol-base ligand in a fac-arrangement, further by two oxygen atoms of a chelating nitrato group. Coordination number six is completed by oxygen atom of a bridging nitrato group. Thus the two Ni(II) centers are doubly bridged: (a) via the common deprotonated phenolato oxygen atom O(1) and (b) via the $\mu$-O,O’ carbonato group, i.e.: Ni(1)-O(2)-N(5)-O(3)-Ni(2). The Ni-N bond distances are in the range from 2.034(2) to 2.124(2) Å, the Ni-O(1) bond lengths vary from 1.965(2) to 2.0296(16) Å. The nitrato groups form a shorter [Ni-O from 2.089(2) to 2.113(2) Å] and a longer Ni-O bond distance [Ni-O from 2.124(2) to 2.240(2) Å]. The octahedra around the metal centers are distorted, with the O-Ni-O bond angles of the chelating nitrato groups showing the largest deviation from rectangular angle [from 69.12(8) to 60.91(7)°]. The Ni(1)-O(1)-Ni(2) bond angles of the $\mu$-phenolato bridges are 121.24(12), 120.83(7), 118.62(8) and 121.45(8)°, for 1 - 4, respectively. The intra-dimeric Ni(1)…Ni(2) distances are 3.4701(8), 3.4751(7), 3.4263(4) and 3.4926(6) Å, and the
shortest inter-dimer metal-metal separation are 6.5175(11), 6.7469(12), 8.0667(5) and 6.5284(9) Å, for 1 - 4, respectively. The Ni-O-N bond angles of the μ-O,O’ carbonato bridges vary from 121.07(18) to 134.84(17)°; the Ni(1)-O(2)…O(3)..Ni(2) torsion angles are +43.6, +46.0, -45.0 and -43.5° for 1 – 4, respectively. Lattice water molecules in 2 and 4 form hydrogen bonds of type O-H…O to oxygen acceptor atoms of nitrato groups (Table 6A.2).

**Figure 6A.4.** (A) Perspective view (30 % probability ellipsoids) of 1 with partial atom numbering scheme. Selected bond distances (Å) and angles (°): Ni(1)-O(1) 2.017(3), Ni(1)-O(2) 2.100(3), Ni(1)-O(5) 2.194(3), Ni(1)-O(6) 2.101(3), Ni(1)-N(1) 2.087(3), Ni(1)-N(2) 2.035(3), Ni(2)-O(1) 1.965(2), Ni(2)-O(3) 2.190(3), Ni(2)-O(8) 2.182(3), Ni(2)-O(9) 2.093(3), Ni(2)-N(3) 2.108(3), Ni(2)-N(4) 2.057(3); O(5)-Ni(1)-O(6) 60.07(11), O(8)-Ni(2)-O(9) 60.13(12), Ni(1)-O(2)-N(5) 123.8(2), Ni(2)-O(3)-N(5) 134.0(2), Ni(1)-O(1)-Ni(2) 121.24(12) (B) Perspective view (30 % probability ellipsoids) of 2 with partial atom numbering scheme. Selected bond distances (Å) and angles (°): Ni(1)-O(1) 1.9744(15), Ni(1)-O(2) 2.2217(19), Ni(1)-O(5) 2.1037(18), Ni(1)-O(6) 2.1242(19), Ni(1)-N(1) 2.117(2), Ni(1)-N(2) 2.051(2), Ni(2)-O(1) 2.0217(16), Ni(2)-O(3) 2.0967(19), Ni(2)-O(8) 2.240(2), Ni(2)-O(9) 2.103(2), Ni(2)-N(3) 2.076(2), Ni(2)-N(4) 2.054(2); O(5)-Ni(1)-O(6) 60.91(7), O(8)-Ni(2)-O(9) 59.12(8), Ni(1)-O(2)-N(5) 123.73(15), Ni(2)-O(3)-N(5) 123.23(16), Ni(1)-O(1)-Ni(2) 120.83(7).
Figure 6A.5. Perspective view (30% probability ellipsoids) of (A) 3 with partial atom numbering scheme; selected bond distances (Å) and angles (°): Ni(1)-O(1) 1.9709(16), Ni(1)-O(2) 2.193(2), Ni(1)-O(5) 2.096(2), Ni(1)-O(6) 2.164(2), Ni(1)-N(1) 2.124(2), Ni(1)-N(2) 2.048(3), Ni(2)-O(1) 2.0133(16), Ni(2)-O(3) 2.097(2), Ni(2)-O(8) 2.215(2), Ni(2)-O(9) 2.113(2), Ni(2)-N(3) 2.070(2), Ni(2)-N(4) 2.057(2); O(5)-Ni(1)-O(6) 60.72(9), O(8)-Ni(2)-O(9) 59.54(8), Ni(1)-O(2)-N(5) 134.84(17), Ni(2)-O(3)-N(5) 121.07(18), Ni(1)-O(1)-Ni(2) 118.62(8) (B) 4 with partial atom numbering scheme; selected bond distances (Å) and angles (°): Ni(1)-O(1) 2.0296(16), Ni(1)-O(2) 2.0936(19), Ni(1)-O(5) 2.106(2), Ni(1)-O(6) 2.199(2), Ni(1)-N(1) 2.079(2), Ni(1)-N(2) 2.034(2), Ni(2)-O(1) 1.9741(15), Ni(2)-O(3) 2.1981(18), Ni(2)-O(8) 2.181(2), Ni(2)-O(9) 2.0866(19), Ni(2)-N(3) 2.111(2), Ni(2)-N(4) 2.064(2); O(5)-Ni(1)-O(6) 59.95(8), O(8)-Ni(2)-O(9) 60.24(8), Ni(1)-O(2)-N(5) 124.35(16), Ni(2)-O(3)-N(5) 133.65(15), Ni(1)-O(1)-Ni(2) 121.45(8).

Thus crystal structure of 3 with the CMe₃ substituted Mannich base shows the shortest Ni(1)⋯Ni(2) distance of 3.4263(4) Å and the smallest Ni(1)-O(1)-Ni(2) bridging bond angle of 118.62(8)° within the series 1–4.
Figure 6A.6. Packing plot of complex-1.

Figure 6A.7. Packing plot of complex-2.

Figure 6A.8. Packing plot of complex-3.
Table 6A.2. Possible hydrogen bond systems for complex-2 and 4.

<table>
<thead>
<tr>
<th>D-H...A*</th>
<th>Symmetry code for A</th>
<th>D...A (Å)</th>
<th>D-H...A (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O(11)-H(91)...O(3)</td>
<td>-x,-y,1-z</td>
<td>3.227(6)</td>
<td>155</td>
</tr>
<tr>
<td>O(11)-H(92)...O(9)</td>
<td>-x,-y,1-z</td>
<td>3.039(6)</td>
<td>155</td>
</tr>
<tr>
<td>Compound 4:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(11)-H(91)...O(4)</td>
<td>1-x,2-y,-z</td>
<td>3.199(6)</td>
<td>145</td>
</tr>
<tr>
<td>O(11)-H(92)...O(5)</td>
<td>1-x,2-y,-z</td>
<td>3.042(5)</td>
<td>151</td>
</tr>
</tbody>
</table>

* D = Donor, A = Acceptor

Structure-property correlation. The structural parameters of the compounds 1-4 correlate with the electronic partial charge (I-effect (inductive effect)) of the substituents in para position of the phenol group within the Mannich base: CMe_3 (3) > CHMe_2 (2) ≈ Me (1) > Cl (4): Ni(1)...Ni(2) distance is 3.4263(4) Å (3) 3.4751(7) Å (2), 3.4701(8) Å (1), and 3.4926(6) Å (4) and Ni(1)-O(1)-Ni(2) angle is 118.62(8)° (3), 120.83(7)° (2), 121.24(12)° (1) and 121.45(8)° (4).

Phosphatase activity. The efficiency of P-O bond cleavage of the dinuclear Ni^{II} complex-1-5 was screened completely by monitoring the spectral change in the wavelength scan of a catalytic solution for 4 h where complex to phosphomonoester is maintained at 1:20 stoichiometrically. The electronically silent substrate 4-NPP (1 × 10^{-3} M) leads to a band maxima at 427 nm characteristic of p-nitrophenolate ion owing to the hydrolytic action of Ni^{II} complex (5 × 10^{-5} M) in aqueous DMF (Figure 6A.10).
Figure 6A.10. Wavelength scan for the hydrolysis of 4-NPP in the absence and presence of complex-1 (substrate:catalyst = 20:1) in DMSO-water at pH 9 recorded at 25 °C at an interval of 10 min for 2 h. [4-NPP] = 1 × 10^{-3} (M), [Complex] = 0.05 × 10^{-3} (M). Arrow shows the change in absorbance with reaction time.

The kinetic treatment of 1-5 has been performed by the conventional initial slope method following the rate of increase in absorption of the band corresponding to [4-nitrophenolate]. The absorbance profile for is portrayed in Figure 6A.11. The kinetic parameters (V_{max}, k_M, k_{cat}) for the catalyzed reactions were determined from the Lineweaver–Burk plots (Figure 6A.12, Table 6A.3). A systematical theoretical modeling employing DFT method is conducted in order to rationalize the above observed trend in phosphatase activity and clarify the plausible reaction mechanisms.
. **Figure 6A.11.** Absorption profile at 420 nm of (A-E) complex-1-5 respectively for the reaction mixture used in wavelength scan. Inset shows the log[Aₓ/(Aₓ-A₀)] vs time plot of the same (R²=0.993, Standard Deviation=0.009, Slope=0.00109, Error=2.56×10⁻⁵).

**Figure 6A.12.** (A) Kinetics plot of complex-1 (Least-squares fit of second order, R²=0.997). Inset shows the Line-weaver Burk plot (R²=0.927, Standard deviation=71.49) (B) Overlay of the Line-weaver Burk plots of complex-1-5 for phosphatase activity.

**Table 6A.3. Kinetic parameters for phosphatase activity of complex-1-5.**

<table>
<thead>
<tr>
<th>Complex</th>
<th>k_{cat} (sec⁻¹)</th>
<th>10⁴ V_{max} (M s⁻¹)</th>
<th>Standard error</th>
<th>10⁻⁴ Km (M)</th>
<th>Standard error</th>
<th>10⁻⁴ k_{assoc} (M⁻¹)</th>
<th>10⁻⁴ k_{cat}/k_{M} (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.66</td>
<td>3.33</td>
<td>8.62×10⁻⁵</td>
<td>5.22</td>
<td>0.016</td>
<td>0.019</td>
<td>1.28</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>14.5</td>
<td>1.17×10⁻⁵</td>
<td>30</td>
<td>0.001</td>
<td>33.33</td>
<td>0.97</td>
</tr>
<tr>
<td>3</td>
<td>80.6</td>
<td>40.3</td>
<td>0.745</td>
<td>69</td>
<td>9.72</td>
<td>0.014</td>
<td>1.17</td>
</tr>
<tr>
<td>4</td>
<td>6.08</td>
<td>3.04</td>
<td>1.14×10⁻⁴</td>
<td>5.62</td>
<td>0.003</td>
<td>0.018</td>
<td>1.08</td>
</tr>
<tr>
<td>5</td>
<td>8.19</td>
<td>4.1</td>
<td>8.92×10⁻⁴</td>
<td>7.6</td>
<td>0.018</td>
<td>0.013</td>
<td>1.08</td>
</tr>
</tbody>
</table>

**Theoretical Modeling.** The constructed catalyst structures are based on the experimental obtained crystal structure.
**a. Basic Consensus.**

As is noted in experimental details, the phosphatase activity of the five Ni$^{II}$ complexes were studied in DMSO- pH 9 buffer (3:1) at room temperature. Therefore, it could be concluded that the metal-bound hydroxide (M-OH) is the nucleophile (Nu), which is commonly acknowledged. The Ni$^{2+}$ center has a d$^8$ electron configuration, which is believed to preferentially adopt a planar square configuration or a polar elongated tetragonal pyramid. The octahedral configuration of Ni$^{2+}$ center in the obtained crystal structure could not be maintained during the reaction coordinate and the planar square configuration or the polar elongated tetragonal pyramid configuration is located instead.

**b. Basic Reaction Mechanism.**

The plausible reaction mechanisms of the hydrolysis of NPP$^{2-}$ catalyzed by [Ni$_2$Ln(OH$^-$)]$^+$ complexes are exclusive. The mechanism in which each phosphoryl oxygen terminal from the substrate is coordinated with the bimetallic center respectively and the bridged hydroxide ion acts as the nucleophile, is no longer available in the case due to the significant planar configuration preference of the Ni$^{2+}$ center and the subsequent severe steric hindrance. Oppositely, a reaction mechanism in which each phosphoryl oxygen atom is also coordinated with the bimetallic center while the nucleophilic reagent hydroxide is only located at one particular Ni$^{2+}$ center, is found to be more plausible. Two reaction modes are proposed based on the mechanism (Figure 6A.13). The major difference between mode 1 and mode 2 is that only the nucleophile coordinated Ni$^{2+}$ center (named as Ni1, the other Ni$^{2+}$ center is named as Ni2) is pentacoordinated while both the bimetallic centers in mode 2 adopt the polar elongated tetragonal pyramid configuration. Both the Ni1 center in mode 1 and 2 play important roles in nucleophile generation (facilitating the deprotonation of water molecule) and substrate activation. However, the Ni2 center in mode 2 not only activate the substrate by forming Ni-O coordination bond but also get involved in the stabilization of leaving group (LG) in transition state by forming a hydrogen bond between the coordinated water molecule and the leaving group. The leaving group will be protonated simultaneously with the deprotonation of the Ni2 coordinated water molecule. It is noted that the pyridyl N atom is coordinated with the Ni2 center in the planar square in mode 1 while located at the elongated polar site of the
tetragonal pyramid configuration of Ni2 in mode 2, the former configuration is believed to be more favorable due to the common sense that pyridyl N is a better coordinate site than the O atom in water molecule. The theoretical results show that the relative free energy of the transition state (Cl-1-H2O-TS, provided below) in mode 1 is 11.4 kcal/mol lower than the transition state in mode 2. Meanwhile, the roles of solvent participation are rationalized by utilizing supermolecule simulation. The optimized structure of Cl-1-H2O-TS shows that the added solvent water molecules play important roles in stabilizing the metaphosphate [PO3]6- planar and stabilizing the leaving group by forming hydrogen bonds. More importantly, Cl-1-H2O-TS obtained from supermolecule simulation is more compacted than Cl-1-TS that the bond length of P-O(Nu) and P-O(LG) in Cl-1-H2O-TS and Cl-1-TS increase from 1.88 Å to 1.91 Å and 2.23 Å to 2.46 Å, respectively. Therefore, the effect of solvent participation could be summarized as stabilizing reaction species, tightening transition states, and involving in proton-transfer relay. The relative free energy of Cl-1-TS is 5.7 kcal/mol higher than that of Cl-1-H2O-TS. Therefore, only mode 1 with solvent participation is considered in the analysis of the influence of para-substitution provide below.

![Figure 6A.13](image)

*Figure 6A.13. Depicted are proposed different reaction modes.*

The proposed reaction mechanisms employing different para-substituted [Ni2Ln(OH-)]+ complexes are all concerted and their optimized structures are very similar. For simplicity, only the chloro substituted entry is provided and their optimized structures and corresponding potential energy surface (PES) profiles are depicted in Figure 6A.14 and 6A.40, respectively. In Cl-1-H2O-RC, the phosphate monoester substrate NPP2- is adequately activated by two significant Ni-O coordination linkages formed between the bimetallic center and two respective phosphoryl oxygen terminals. The electron-deficient
phosphorous center could be nucleophilically attacked by the metal-bound hydroxide ion and the transition state Cl-1-H2O-TS is obtained. Both P-O(Nu) bond formation and P-O(LG) fission processes are observed when checking the vibratory movements of the unique imaginary frequency of the transition state. Finally, the P-O(LG) bond is cleaved and the leaving group is dissociated into solution in the product Cl-1-H2O-PC. Therefore, the hydrolysis mechanism of NPP$^2-$ catalyzed by each [Ni$_2$Ln(OH$^-$)]$^+$ complex is a S$_N$2-type associative concerted mechanism (IUPAC recommended nomenclature is A$_N$D$_N^{11}$). As can be seen from Figure 6A.15, the relative free energy barrier of the Cl-substituted case is 15.6 kcal/mol which is in good agreement with the experimental result ($k_{cat} = 6.08 \text{ s}^{-1}$ and the corresponding activation energy is 16.4 kcal/mol). Therefore, it can be concluded that our proposed reaction mechanism is very feasible.

**Figure 6A.14.** Depicted are optimized structures and the labels of the atoms in the key reaction section of the Cl-substituted case.

**Figure 6A.15.** Depicted are the PES profiles of the Cl-substituted case.
c. The Influence of para-Substitution.

The hydrolysis of NPP\(^{2-}\) mediated by five different para-substituted binuclear Ni\(^{ll}\) complexes in the manner of mode 1 employing supermolecule simulation were conducted to rationalize the influence of para-substitution and the results are provided in Table 6A.4. The experimental values of activation free energies are very close (within 1.5 kcal/mol) which is corresponding to the unconspicuous change of the electronic effect of the para-substituted functional group. Similarly, the theoretical relative free energy barriers obtained in each case are also very close (within 1.9 kcal/mol). Despite of the subtle energetic distinctions, the theoretical results and experimental observation keep consistent in the trend that the electron-donating group (methyl, isopropyl, tert-butyl, methoxy et al.) decreases the reaction energy barrier while electron-withdrawing group (chloro for instance) heightens the energy barrier. The distinctions of the free energy barriers sequence in electron-donating groups between the experimental and theoretical results could be ascribed to the computational errors, because the $\sigma_p$ values of these utilized electron-donating groups are very close (in the range of -0.27 to -0.15) which results in a consequent unconspicuous change of the electronic effect and finally subtle free energy differences.

Table 6A.4. First order rate constant values, $\sigma_p$ values, experimental activation free energies and theoretical relative free energy barriers for phosphatase activity.

<table>
<thead>
<tr>
<th>Complex</th>
<th>1 (Methyl)</th>
<th>2 (Isopropyl)</th>
<th>3 (Tert-butyl)</th>
<th>4 (Chloro)</th>
<th>5 (Methoxy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ (sec(^{-1}))</td>
<td>6.66</td>
<td>29.00</td>
<td>80.60</td>
<td>6.08</td>
<td>8.19</td>
</tr>
<tr>
<td>$\sigma_p$</td>
<td>-0.17</td>
<td>-0.15</td>
<td>-0.20</td>
<td>0.23</td>
<td>-0.27</td>
</tr>
<tr>
<td>$\Delta G^\neq_{Exp.}$</td>
<td>16.3</td>
<td>15.5</td>
<td>14.9</td>
<td>16.4</td>
<td>16.2</td>
</tr>
<tr>
<td>$\Delta G^\neq_{Calc.}$</td>
<td>14.0</td>
<td>13.7</td>
<td>14.4</td>
<td>15.6</td>
<td>14.7</td>
</tr>
</tbody>
</table>

a: the experimental values of activation free energy (in kcal/mol), $\Delta G^\neq = -RT\ln(k_{cat}/k_B T)$, $R=8.3145$ J·mol\(^{-1}\)·K\(^{-1}\)$, $T=298.15$K, $h=6.626\times10^{-34}$ J·s, $k_B=1.381\times10^{-23}$J·K\(^{-1}\). b: the theoretical values of the relative free energy barriers (in kcal/mol).
What is the principle behind the inductive effect of the para-substitution? Neves et al. and Gahan et al. suggested that the para-substituent influenced the acidity of M-O H₂ group but their small range of pKₐ values and inherent errors make a similar analysis more problematic. The natural population analysis (NPA) charge obtained from NBO calculation shows that the partial charge on Ni1 and Ni2 do not change with the different para-position substituent. Only the partial charge on the phenolate oxygen atom (labeled as O6, seen in Figure 6A.39) is distinctly varied with the substitution change. Meanwhile, little variations in the bond length of Ni2-O6, Ni1-O6 and bimetallic distance Ni1---Ni2 are observed in the optimized structures with different para-substitution. Hence, the inductive effect of the para-substitution does not result in obvious structural deformation and different charge population. Neves and coworkers found that a linear correlation was obtained by plotting the energies of α-HOMO orbitals of different para-substituted complexes against the Hammett parameter (σₚ) for the substituents. Similarly, a molecular orbital analysis was conducted on each optimized reactant complex and the selected Kohn–Sham frontier orbitals are provided in Figure 6A.16a. The key reactive orbitals are HOMO-2 and LUMO orbitals (LUMO+1 in chloro-substituted entry). Both the π-orbitals from phenolate group (including the contribution of the para-position substituents) and d orbitals from the bimetallic Ni²⁺ center contribute to the HOMO-2 orbital. The d orbitals have mainly dz² character from Ni1 center and dxy character from Ni2, which are corresponding to the elongated Ni1-O6 axially coordination linkage and the Ni2-O6 bond in the planar square, respectively. A linear correlation is also obtained by plotting the energies of HOMO-2 orbitals in each substituted entry against each corresponding σₚ value (Figure 6A.16b). The heterolytic fission of P-O(LG) indicates an electron withdraw from the phosphate to the leaving group and furthermore, electron-donating groups at the para-position of the phenolate could facilitate this electron withdraw. Both the π-orbitals from the leaving group and d orbital from the Ni2 center (mainly dxy character) contribute to the LUMO orbitals (LUMO+1 for chloro-substituted case). The molecular orbital overlap between HOMO-2 and LUMO is the dxy orbital of Ni2 center and this particular dxy orbital from Ni2 center is speculated to bridge the electron withdraw process. Interestingly, a linear correlation is obtained by plotting the energy gap between LUMO (LUMO+1 for Cl-substituted) and HOMO-2 orbitals for each optimized reactant complex against the
corresponding \( \sigma_p \) value (seen in Figure 6A.15c). As can be seen from Figure 6A.40b and c, higher Hammett parameter \( \sigma_p \) value results in lower HOMO-2 orbital and larger energy gap between the LUMO and HOMO-2 orbitals. Therefore, the influence of para-substitution could be concluded that the electron-donating group decreases the reaction energy barrier via reducing the energy gap between the orbital of electron-sufficient para-substituted phenolate group and the electron-demanding leaving group.

Figure 6A.16. Depicted are the LUMO (LUMO+1 in chloro-substituted entry) and HOMO-2 orbitals for each optimized reactant complex (a) and linear correlation from Hammett parameter (\( \sigma_p \)) and energies of HOMO-2 orbitals (b) and energy gaps between LUMO and HOMO-2 orbitals (c).
Catecholase activity. The catecholase activity of the five Ni\textsuperscript{II} complexes were examined against 3,5-DTBC in DMSO-MeOH solvent and the results showed moderate catechol oxidation (Figure 6A.17 for complex-1). Their corresponding enzymatic kinetics plot was obtained by Michaelis Menten analysis as shown in Figure 6A.18A and from the overlay of the double reciprocal plots it is evident that complex-3 has the highest catalytic efficiency (Figure 6A.18B, Table 6A.5).

**Figure 6A.17.** Wavelength scan of a mixture of DTBC and complex-1 in DMSO-MeOH solvent mixture for 2 hours at an interval of 5 minutes showing catecholase activity.

Conditions: [DTBC] = 10^{-2} (M), [complex-1] = 10^{-4} (M), Temperature = 298 K.

**Figure 6A.18.** Kinetics plot (A-E) of complex-1-5 respectively. Inset shows the Line-weaver Burk plot (B) Overlay of the Line-weaver Burk plots for the catecholase activity of 5 complexes.
CHAPTER 6A

Table 6A.5. Kinetic parameters for phosphatase activity of complex-1-5.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$10^4 \times k_{cat}$ (sec$^{-1}$)</th>
<th>$10^3 V_{max}$ (M s$^{-1}$)</th>
<th>$10^6$ Standard error</th>
<th>$10^4$ $K_m$ (M)</th>
<th>$10^4$ Standard error</th>
<th>$10^4 k_{assoc}$ (M$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.7</td>
<td>4.83</td>
<td>0.02</td>
<td>10.71</td>
<td>2.07</td>
<td>0.093</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>7.6</td>
<td>7.62</td>
<td>0.047</td>
<td>18.9</td>
<td>0.25</td>
<td>0.053</td>
<td>0.403</td>
</tr>
<tr>
<td>3</td>
<td>11.4</td>
<td>11.36</td>
<td>1.485</td>
<td>20.28</td>
<td>0.70</td>
<td>0.05</td>
<td>0.56</td>
</tr>
<tr>
<td>4</td>
<td>4.11</td>
<td>4.11</td>
<td>0.133</td>
<td>13.27</td>
<td>9.6</td>
<td>0.075</td>
<td>0.31</td>
</tr>
<tr>
<td>5</td>
<td>4.39</td>
<td>4.39</td>
<td>0.075</td>
<td>5.35</td>
<td>0.035</td>
<td>0.19</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Complex mediated cytotoxic effect on HepG2 cells. Inhibition of cell proliferation was examined by incubating cells with different concentrations of di nuclear Ni$^{II}$ complex and carboplatin as a standard for 24 hr and 48 hr. Human hepatocellular carcinoma cells (HepG2 cell line) were treated with the complexes and carboplatin in a dose dependent manner (10, 20, 40, 60, 80 μM) (Figure 6A.19A). These complexes have a significant inhibitory effect on the proliferation of HepG2 cells. IC$_{50}$ values were significantly lower in the present case than that exhibited by carboplatin as tested under similar conditions. Complex-3 was found to be the most cytotoxic complex, with IC$_{50}$ values of 34.58 μM for 24 hr and 26.3 μM for 48 hr against HepG2 cells. In terms of reduction of cell viability, the compounds rank HepG2 cells in the order Complex-3>Complex-4>Complex-1>Complex-5>Complex-2 for 24 hr and Complex-3>Complex-5>Complex-4>Complex-1>Complex-2>Carboplatin for 48 hr. All these data clearly suggests that the presence of a metal ion in the complex is required for the cell toxicity. Based on the cytotoxicity we selected complex-3 for further studies to observe its mechanism of cytotoxic action.

Complex-3 increased LDH activity and cellular NO level. To further confirm the cytotoxic potential of complex-3, HepG2 cells were treated with complex-3 (40 μM) in time dependant manner (8 hr, 16 hr and 24 hr). The result showed that a time dependant release of LDH (Figure 6A.19B) and NO (Figure 6A.19C) in the culture medium, indicating the cytotoxic effect of complex-3 on liver cancer cells.
**Complex-3 increased lipid peroxidation.** A significant (0<0.05) time dependent increase in lipid peroxidation, measured as TBARS formation, was observed in complex-3 treated HepG2 cells (Figure 6A.19D) with the maximum value observed at 40 μM complex-3 treatment for 24 hr compared to control cells.

![Graphs showing cellular viability and toxicity](image)

**Figure 6A.19.** Cytotoxic effect of nickel (II) complex on HepG2 cells. (A) Nickel (II) complex (1, 2, 3, 4 and 5) inhibited proliferation of HepG2 cells in a dose and time dependent manner: Cell viability after treatment with different concentrations of nickel (II) complex (10, 20, 40, 60 and 80 μM) for 24 and 48 hr was assessed by MTT cell proliferation assay. The results were expressed as % cell viability. (B) Cytotoxic effect of nickel (II) complex-3 on cultured HepG2 cells: LDH leakage after nickel (II) complex-3 (40 μM) treatment for 8, 16 and 24 hr was measured to assess cytotoxicity. (C) Nickel (II) complex-3 treatment increased nitric oxide release: Nitric oxide release after nickel (II) complex-3 (40 μM) treatment for 8, 16 and 24 hr was measured. (D) Nickel (II) complex-3 treatment increased lipid peroxidation: TBARS formation after nickel (II) complex-3 (40 μM) treatment for 8, 16 and 24 hr was measured. Values are presented as mean ± SEM (n=5). p<0.05 was considered as significant. Statistical comparison: *Control vs. Nickel (II) complex-3 treated group (8 hr), **Control vs. Nickel (II) complex-3 treated group (16 hr), ***Control vs. Nickel (II) complex-3 treated group (24 hr).
CHAPTER 6A

**Complex-3 induced apoptosis.** To get the insight of the diminishment of cell viability, apoptotic pathway was investigated. The apoptosis inducing ability of complex-3 was measured by Annexin-FITC/PI staining. The results showed that complex-3 induced significant apoptosis in HepG2 cells in time dependent manner (Figure 6A.20A). Especially after complex-3 (40 μM) treatment for 24 h, the early apoptotic cells (right lower domain of the fluorocytogram) and late apoptotic cells (right upper domain of the fluorocytogram) increased from 2.18 ± 0.61 % and 1.50 ± 0.34 % to 14.62 ± 0.84 % and 66.16 ± 3.11 %, respectively, thus, the total apoptotic rate reached 80.79 % on average for the 40 μM complex-3 treated cells (Figure 6A.20B). The results suggested that complex-3 induced apoptosis of HepG2 cells in a time dependent manner.

![Figure 6A.20. Representative flowcytogram of annexin V binding (Horizontal axis) versus propidium iodide (PI) uptake (Vertical axis) of HepG2 cells treated with nickel (II) complex-3 (40 μM) for 8, 16, 24 hr. Quantitative analysis of different phase of apoptosis were represented from three independent experiments. Values are presented as mean ± SEM.](image)

**Complex-3 induced activation of γ-H2AX (Ser-139).** Phosphorylation of histone H2AX (γ-H2AX) at Ser-139 indicated cellular responses to genotoxic stress, especially DNA double-strand breaks. To elucidate the possible mechanism of Complex-3 induced apoptosis, γ-H2AX foci formation was detected after 8 hr, 16 hr and 24 hr of post treatment. The results from immunofluorescence staining showed an increased formation of γ-H2AX foci in Complex-3 treated HepG2 cells at 24 hr (Figure 6A.21). However, at 8
hr and 16 hr, γ-H2AX foci formation was significantly increased compared to control cells indicating time dependant DNA damage with Complex-3 in HepG2 cells.

Figure 6A.21. Nickel (II) complex-3 induced DNA damage: Effect of nickel (II) complex-3 (40 μM) on induction of γ-H2AX foci as measured by immunocytochemistry.

**Complex-3 induced accumulation of p53.** p53 is one of the most powerful tumor suppressor genes in human cancers. Earlier reports demonstrated the direct correlation with DNA damage leading to activation of p53, cell cycle arrest and apoptosis. To confirm the transcription independent induction of mitochondrial pathway by p53 is involved in complex-3 induced apoptosis, immuno-fluorescent staining was performed. The accumulation of p53 was confirmed by the co-localization of p53-FITC fluorescent signals in the cytosol of complex-3 treated cells compared to control (Figure 6A.22A).

**Complex-3 led to up-regulation of Bax and down-regulation of Bcl-2.** Bcl-2 family proteins are crucial for apoptosis commitment, mainly via the control of the mitochondrial pathway which is frequently triggered in response to chemotherapeutic agents whereas Bax is the pro-apoptotic factor and act as sensors of specific types of
cellular stress and critical downstream mediators of p53 signaling. To investigate the cellular mechanism underlying complex-3 induced apoptosis in HepG2 cells, the expression of apoptosis-regulated proteins; including pro-survival Bcl-2 and pore-forming Bax was analysed using immunoblot. As illustrated in Figure 6A.22B, the expression level of Bcl-2 was gradually down-regulated after the treatment of complex-3 in time dependant manner (8 hr, 16 hr and 24 hr). In addition, complex-3 significantly induced the activation of Bax in time dependant manner (8 hr, 16 hr and 24 hr) (Figure 6A.22C). To further confirm the down regulation of Bcl2 protein in complex-3 treated HepG2 cells, immunofluorescence study was evaluated. The result showed that mean FITC fluorescence was increased at 24 hr after the treatment of complex-3 (40 μM) compared to control cells (Figure 6A.23A). These results indicated that complex-3 leads to a shift from anti-apoptosis to pro-apoptosis by altering the function of the proteins in the Bcl-2 family.

**Complex-3 increased caspase-3 and caspase-9 activity.** Both caspase-3 and 9 are key enzymes involved in the apoptotic process by activating a number of apoptotic proteins (such as caspase activated DNase and Rho-kinase I) through enzymatic cleavage of the proteins. As shown in Figure 6A.48 at 24 hr after treatment of complex-3 (40 μM) significantly (p<0.05) increased caspase-3 and 9 activities compared to control (Figure 6A.22D). The result also showed that treatment with 40 μM of complex-3 gradually increased caspase-3 and caspase-9 activities at 8 hr and 16 hr compared to control cells. Thus, the results confirmed the involvement of activation of caspases in complex-3 induced apoptosis of HepG2 cells.
Figure 6A.22. Nickel (II) complex-3 modulated the expression of apoptotic proteins. (A) Immunofluorescence micrographs of enhanced expression of p53 along with the down regulation Bcl2. (B) Immunoblot analysis of enhanced expression of Bax and down regulation of Bcl2 protein after treatment with nickel (II) complex-3 (40 μM) for 8, 16 and 24 hr. β-Actin was used as internal control. (C) Densitometric analysis of respective protein expression on the basis of relative intensity of immunoblot. (D) Activities of caspase-3 and caspase-9 after treatment of nickel (II) complex-3 (40 μM) for 8, 16 and 24 hr. Values are presented as mean ± SEM (n=5). p<0.05 was considered as significant. Statistical comparison: *Control vs. Nickel (II) complex-3 treated group (8 hr), **Control vs. Nickel (II) complex-3 treated group (16 hr), ***Control vs. Nickel (II) complex-3 treated group (24 hr).

Complex-3 increased loss of mitochondrial membrane potential (ΔΨm). The integrity of the mitochondrial membrane potential (ΔΨm) is important for cell survival. The loss of ΔΨm may induce the apoptosis. To confirm the intrinsic apoptosis induced by complex-3 treatment, ΔΨm was detected by flow cytometry analysis using mitochondrial specific probe JC-1 after the cells were treated with complex-3 (40 μM). The result showed that the mitochondrial membrane depolarization was initially observed at 8 hr after exposure to 40 μM of Complex-3, and continuously shifted to the right in a time dependent manner, indicating that the collapse of MMP was highest at 24 hr (Figure 6A.23). Thus the result suggested that complex-3 was capable of inducing mitochondrial depolarization in a time-dependent manner, and that MMP loss may be directly correlated with apoptosis.

Figure 6A.23. Nickel (II) complex-3 disrupted mitochondrial membrane potential (ΔΨm) in HepG2 cells. A time dependent (8, 16 and 24 hr) loss of (ΔΨm) was observed in HepG2
cells after exposure to nickel (II) complex-3 (40 μM) as evidenced by diminution of JC-1 red fluorescence to green fluorescence.

Complex-3 induced apoptosis through the generation of reactive oxygen species (ROS) and cytosolic Ca$^{2+}$ levels. As reactive oxygen species (ROS) plays an important role in apoptosis, we investigated the ability of complex-3 to generate ROS in HepG2 cells. Cells were treated with complex-3 (40μM) for 8 hr, 16 hr and 24 hr respectively to analyze the generation of ROS by flow cytometry (Figure 6A.24A). The result showed that the generation of ROS by complex-3 was increased significantly ($p<0.05$) in time-dependent manner (Figure 6A.24B). To examine the direct involvement of complex-3 generated ROS in apoptosis of HepG2 cells, cell viability study was done after treatment of complex-3 (40 μM) with or without NAC. NAC is a potent ROS scavenger that can inhibit oxidative stress by directly scavenging ROS and replenishing GSH. If ROS production interfered with complex-3 induced cell death, NAC should have the ability to inhibit complex-3 induced cell death. The result showed that complex-3 (40μM) decreased the cell viability to 62.59 %, 50.25 % and 38.65 % at 8 hr, 16 hr and 24 hr whereas suppressing the complex-3 mediated ROS by NAC led to restore the cell viability to 85.45 %, 86.72 % and 90.17 % (Figure 6A.24C). Ca$^{2+}$ reached the highest after the induction with complex-3 (40 μM) for 24 hr (Figure 6A.24D).

Figure 6A.24. Nickel (II) complex-3 induced oxidative stress in HepG2 cells. (A) Nickel (II) complex-3 induced intracellular ROS accumulation in HepG2 cells: Treatment of HepG2 cells
with nickel (II) complex-3 for 8, 16 and 24 hr resulted in hyper-generation of reactive oxygen species. (B) Quantitative analysis of relative DCF fluorescence intensity obtained from respective flowcytogram. (C) Cell viability after treatment with or without different concentrations of nickel (II) complex (10, 20, 40, 60 and 80 μM) and NAC (10 mM) for 8, 16 and 24 hr. (D) Effect of nickel (II) complex-3 on intracellular Ca\(^{2+}\) concentration: Treatment with nickel (II) complex-3 (40 μM) increased intracellular Ca\(^{2+}\) levels in a time dependent (8, 16 and 24 hr) manner. The results were expressed as relative Fura 2/AM fluorescence.

**Complex-3 reduced cellular antioxidant status.** The level of non-enzymatic antioxidant GSH was found to be decreased concomitantly with the reactive oxygen species generation (Figure 6A.25A). The activities of enzymatic antioxidants such as SOD (Figure 6A.25B) and CAT (Figure 6A.25C) were found to be significantly decreased in HepG2 cells incubated with complex-3 when compared to control.

![Figure 6A.25](image)

*Figure 6A.25. Nickel (II) complex-3 lowered antioxidant enzymes activity. HepG2 cells were treated with nickel (II) complex-3 (40 μM) for 8, 16 and 24 hr. The cell lysate were analysed for (A) GSH, (B) SOD and (C) CAT activities. Values are presented as mean ± SEM (n=5). p<0.05 was considered as significant. Statistical comparison: *Control vs. Nickel (II) complex-3 treated group (8 hr), **Control vs. Nickel (II) complex-3 treated group (16 hr), ***Control vs. Nickel (II) complex-3 treated group (24 hr), NS=non significant.*

Apoptosis plays an elementary role in many pathophysiological processes in the liver.\(^{30}\) Induction of apoptosis in cancer cells is therefore regarded as one of the possible approaches for treatment of human tumors. However, the mechanism responsible for
apoptosis induced by nickel (II) complex-3 is not known. The results of the present study indicated that various nickel (II) complex-3 inhibited cell proliferation and induced apoptosis in HepG2 cells in dose (0-80 μM) and time (8 hr, 16 hr, 24 hr and 48 hr) dependent manner. Decrement of cell viability caused by nickel (II) complex in dose and time dependently encouraged us to further investigate whether there was the intervention of apoptosis with the exploration of mechanism involved. With the increase of time (8 hr, 16 hr and 24 hr), 40 μM of complex-3 significantly decreased the cell viability, followed by increased the apoptosis. LDH is a stable cytoplasmic enzyme that is released into the culture medium following loss of membrane integrity and serves as a general means to assess cytotoxicity resulting from chemical compounds. The significant increase of LDH level in the culture supernatant confirmed the cytotoxic effect of complex-3 on liver cancer cells. Treatment of HepG2 cells with nickel (II) complex-3 (40 μM) resulted in a time dependent increase of NO release. Earlier studies showed that genotoxic stress lead to the activation of iNOS which in turn releases NO. The nitrite is the stable product of the nitric oxide released in response to oxidative stress, the amount of nitrite in the culture medium corresponds to the level of nitric oxide. Hence the level of nitrite is estimated to measure the NO produced after complex-3 treatment. NO is a gaseous signaling molecule and a well-known, short-lived free radical which is produced non-enzymatically by iNOS and causes damage to most biomolecules, including DNA and protein. The apoptotic rate of HepG2 cells was quantified using annexin V and PI dual staining by flow cytometry. The proportional increase of annexin V+/PI+ cells induced by complex-3 was significant, suggesting the apoptotic process. The results were consistent with the earlier reports. Cytotoxic agent may induce the depolarization of the mitochondrial membrane, which in turn results in the activation of mitochondria-mediated apoptosis pathway by the release of mitochondrial pro-apoptotic factors. A rapid collapse of ΔΨm results in mitochondria mediated apoptosis in cancer cells. Our data clearly demonstrated that treatment with 40 μM of complex-3 could lead to a loss of mitochondrial transmembrane potential in time dependent manner. This means that complex-3 induced apoptosis is related to the collapse of ΔΨm. The collapse of ΔΨm implies the opening of mitochondrial permeability transit pore. In addition, down-regulated expression of the apoptosis-regulating protein Bcl2 and over-expression of Bax was observed in a time dependent manner (8 hr, 16 hr and 24 hr)
with the treatment of complex-3 (40 μM). It was further confirmed that complex-3 had a proapoptotic effect. Induction of p53 has also been shown to produce changes in redox metabolism, leading to increases in DNA double strand break prior to the onset of apoptotic cell death.\textsuperscript{35} A body of work indicated that p53-mediated apoptosis proceeds primarily through the intrinsic apoptotic program.\textsuperscript{36} It was reported that p53 induced apoptosis by either increasing transcriptional activity of proapoptotic genes such as Bax or suppressing the activity of the antiapoptotic gene of the Bcl2 family. Thus, in order to determine whether the ability of nickel (II) complex-3 in inducing apoptosis is due to the effect of p53, changes in the expression of p53 were also examined. The results indicated that complex-3 exposure to HepG2 cells could enhance the expression of p53. Bax can directly cause mitochondria to release cytochrome c. Cytochrome c is released from mitochondria into cytosol, responsible for activating caspase-9, which further activates caspase-3 and executes the apoptotic program. Thus, we checked the direct apoptosis effect in the protein levels of caspase-9 and the activity of caspase-3. Nickel (II) complex-3 (40 μM) exposure increased the activity of caspase-9 and caspase-3 time dependently. The increment of caspase-3 and decrement in Bcl-2 level can be explained by that Bcl-2 was cleaved and converted to proapoptotic proteins such as Bax. Moreover, the apoptosis, induced by nickel (II) complex-3, may be triggered via caspase-9 pathway. It has been reported that the activation of caspase appeared to be largely mediated by ROS generation. Accumulating data has reported that ROS can lead to the overload of Ca\textsuperscript{2+} concentration in cells. The increment of ROS production and cellular Ca\textsuperscript{2+} level was observed in a time-dependent manner. The highest level of Ca\textsuperscript{2+} was 24 h post treatment by complex-3, while the highest production of ROS also was 24 h after the complex-3 treatment. The increment of ROS was earlier than that of Ca\textsuperscript{2+} overload; suggesting that the increment of Ca\textsuperscript{2+} concentration might be due to the stimulation of ROS. Thus, a time-dependent increase in ROS levels in HepG2 cells, suggested that ROS may be a critical initiator of nickel (II) complex-3 induced apoptosis. Mitochondria are the major source of ROS production. Mitochondria damage leads to excessive ROS release, which may further damage mitochondria and cause the loss of mitochondrial membrane potential (ΔΨm) to initiating the apoptosis process. The results of the present study revealed the fact that nickel (II) complex-3 reduced the cellular antioxidant pool concomitantly with an increase
in ROS generation. In this experiment, the antioxidant enzyme activities in the untreated HepG2 cells were higher compared to complex-3 treated counterparts. This might be due to the hyper generation of ROS, which in turn converts the reduced glutathione to its oxidized form. Increased ROS has a direct effect on SOD and CAT. Moreover a significant increase in the lipid peroxide levels in HepG2 cells after complex-3 treatment, which might be due to the hyper generation of ROS. Our results are in line with the previous reports. The depleted GSH pool and increased reactive oxygen species production in HepG2 cells were accompanied by an increase in lipid peroxidation, which presumably plays a critical role in oxidative stress-induced mitochondrial dysfunction and apoptosis.

6A.4. Conclusion

In conclusion these results demonstrate for the first time that apoptosis is the mechanism implied in nickel (II) complex exposure (Figure 6A.26). Data obtained in the present study would help to illustrate the mechanism of cancerous cell death induced by nickel complex-3 and to look for a novel treatment for liver cancer.

Figure 6A.26. Schematic representation of complex-3 mediated apoptosis.
6A.5. References


(10) (a) Bruker 2005 SAINT v. 7.23.; APEX, Bruker AXS Inc., Madison, Wisconsin, USA; (b) Sheldrick, G.M. 2001, SADABS v. 2. University of Goettingen, Germany.


(26) Moron, M.S.; Depierre, J.W.; Mannervik, B. *Biochimica et Biophysica Acta* 1979, 582, 67-78.


SYNTHESIS, CHARACTERISATION AND CATALYTIC STUDY OF A SCHIFF-BASE NICKEL CORE: A VERSATILE BIOINSPIRED METALLOCATALYST
Abstract

A versatile metallocatalyst \([\text{Ni}_2L_2(\text{NCS})(\text{Ac})(\text{H}_2\text{O})_{0.5}(\text{MeOH})_{0.5}] \cdot 1.25\text{H}_2\text{O}\) (HL = 2-((E)-(2-(pyridin-2-yl)ethylimino)methyl)-4-chlorophenol) has been synthesized and characterized by single crystal XRD and standard physicochemical techniques. It portrays catecholase activity as an oxygen dependent enzymatic radical catalysis under completely aerobic conditions \((\lambda_{\text{max}} = 375 \text{ nm}, \varepsilon = 1900 \text{ M}^{-1} \text{ cm}^{-1})\) against the model substrate 3,5-DTBC. The phosphatase activity of the complex has been analysed on the monophosphoester 4-NPP where the gradual release of 4-nitrophenolate is monitored spectrophotometrically \((\lambda_{\text{max}} = 400 \text{ nm}, \varepsilon = 18500 \text{ M}^4 \text{ cm}^{-1})\). Michaelis-Menten analysis of pseudo first-order reaction kinetics establishes that DMF medium provides a better catalytic pathway for catecholase activity \((k_{\text{cat}} = 2.8 \times 10^{-3} \text{ s}^{-1})\) than acetonitrile \((9.11 \times 10^{-4} \text{ s}^{-1})\) while phosphate-bond hydrolysis occurs only in DMF medium \((8.18 \text{ s}^{-1})\), all under excess substrate conditions. Antileukemic efficacy was tested towards KG-1A (AML) and K562 (CML) cell lines. Cell viability study, drug uptake assay, ROS formation, alteration of MMP, apoptosis study, DNA fragmentation, DAD assay, MIC and MBC level testing portrays that the complex can produce a significant dose dependent antileukemic property on both cell lines.

Keywords: Dinuclear Ni(II); Schiff-base; Catecholase; Phosphatase; Biological activity.
6B.1. Introduction

Nickel(II), a bio-essential trace element,\textsuperscript{1-3} sketches a pioneering coordination chemistry owing to its multi-flexible nature associated with configurational switch. Recent developments in the field of nickel bio-chemistry and its catalytic applications provide huge thrust in this area.\textsuperscript{4-8} Nickel complexes showing catecholase activity,\textsuperscript{9-14} one of the three dicopper proteins (type 3) which exclusively catalyzes the oxidation of catechols to their corresponding o-quinones without acting on monophenols, have also been reported based on metal-centred oxidation and a radical pathway. Throughout all these works, the catalytic influence of various stereo-electronic factors on the catecholase activity has been analyzed, but until now there are no concluding results that could single out the solvent effect in nickel system. Likewise, phosphatase activity, although a characteristic of zinc complexes\textsuperscript{15}, have also been remarkably exhibited by other metal ions,\textsuperscript{16-23} more so for dinuclear complexes since thermodynamic driving force for redox reactions is lowered due to charge-delocalization along with the activation barrier for solvent-enzyme reorganization.\textsuperscript{24} Starting from this point, our group had taken up the synthesis, structural characterization and investigation of catecholase, phosphatase and biological activity of a dinuclear nickel(II) complex derived from a phenolic Schiff-base ligand.\textsuperscript{25} Recent drug development study involving metal-based anticancer drugs began with \textit{cis}-platin invention, a Pt(II) complex, by Rosenberg in the 1960s.\textsuperscript{26} Schiff-base complexes, having chelating and intermetallating property, are excellent for anti-bacterial, anti-fungal and anti-tumor activity\textsuperscript{27,28} other than therapeutic applications.\textsuperscript{29} Like Mn\textsuperscript{II} complexes,\textsuperscript{30} the present study was conducted on our Ni\textsuperscript{II} complex to explore the anti-leukemic and anti-bacterial activity on leukemic cell lines (KG-1A and K562) and multi drug resistant E. coli and S. aureus bacteria.

Thus on the path to develop synthetic analogues of metalloenzymes, we have synthesized a small dinuclear nickel(II) complex of 2-\((E)-(2-(pyridin-2-yl)ethylimino)methyl)-4-chlorophenol (Scheme 6B.1) to probe the structure, function and spectroscopic properties of catechol oxidase and metallohydrolases.
6B.2. Experimental section

Materials and Methods. All reagents were of the highest grade commercially available and were used without further purification. 5-chlorosalicylaldehyde and 2-(2-Pyridylethyl)amine was purchased from Sigma-Aldrich. Nickel acetate, sodium thiocyanate, magnesium sulphate and brine were obtained from Merck. Elemental analyses (carbon, hydrogen and nitrogen) were performed using a Perkin-Elmer 240C analyzer. Infrared spectra (4000–400 cm$^{-1}$) were recorded at 28 °C on a Shimadzu FTIR-8400S using KBr as a medium. Electronic spectra (800–200 nm) were obtained at 25 °C using a Shimadzu UV-3101 PC, where dry acetonitrile and DMF was used as a medium as well as a reference. EPR experiments were performed at liquid nitrogen temperature (77 K) in acetonitrile using a JEOL JES-FA200 spectrometer at X band (9.13 GHz). Thermal analyses (TG–DTA) were carried out on a Mettler Toledo (TGA/SDTA851) thermal analyzer in flowing dinitrogen (flow rate: 30 cm$^3$ min$^{-1}$). Cyclic voltammetric and DPV measurements were performed by using a CH1106A potentiostat with glassy carbon (GC) as working electrode, Pt-wire as counter electrode and Ag,AgCl/sat KCl as reference electrode. Potentials are uncorrected for any junction contributions. All solutions were purged with dinitrogen prior to measurements Spectral data (UV-vis/NIR) were obtained using a Perkin-Elmer Lambda 750 spectrophotometer.
Synthesis of ligand. To a 10 ml ethanolic solution of 5-chlorosalicylaldehyde (1 mmol, 156 mg), 5 ml of 2-(2-Pyridylethyl)amine (1 mmol, 122 mg) in ethanol was added dropwise with stirring over a period of 30 minutes. It was refluxed for two hours and then the solution was evaporated under reduced pressure. The resultant yellow oil was extracted with diethyl-ether and saturated-brine solution several times. The organic phase was separated, dried with anhydrous MgSO₄, concentrated by evaporation of ether and subsequently vacuum- dried for the removal of last traces of water. An yellow crystalline solid was obtained from it. Yield=105 mg (40.3%).

Elemental analysis calcd (%) for C_{14}H_{13}N₂ClO: C 64.49, H 5.03, N 10.74, O 6.14; found C 64.98, H 5.01, O 5.92 Cl 13.42. ¹H NMR (300MHz, CDCl₃, 25°C) : δ= 13.311 (s, 1H; phenolic O-H), 8.490-8.471 (d, 1H; N-CH-CH of pyridine), 8.127 (s, 1H; imine H), 7.548-7.535 (t, 1H; N-CH-C-H of pyridine), 7.156-7.147 (d, ortho to phenolic O-H), 7.118 (s, 1H; ortho to phenolic O-H and C=N), 7.108-7.084 (d, 1H; N-C=CH), 7.071-7.044 (t, 1H; para to pyridine N atom), 6.803-6.774 (d, 1H; C(Cl)-CH-C), 3.982-3.936 (t, 2H; CH₂-Py ring), 3.119-3.072 (t, 2H; CH₂-imine). ¹³C NMR (300MHz, CDCl₃, 25°C) : δ= 164.11 (substituted C ortho to pyridine N), 159.74 (unsubstituted C ortho to pyridine N), 158.78 (C-OH), 149.47 (C-Cl), 136.43 (C=C=N), 131.95 (C-C-OH), 130.26 (Cl-C=C-C=C=N), 123.64 (Cl-C-C), 122.97 (Npy-Csubs-C), 121.56 (Npy-Cunsubs-C), 119.37 (para to Npy), 118.51 (C=N), 58.87 (C=N=CH₂), 39.17 (C=N-Ch₂=CH₂). FT-IR data (KBr pellet): γbar= 1634 (C=N), 1593 (benzene ring skeletal), 1480 (pyridine ring skeletal) cm⁻¹.

Synthesis of metal-complex. 1 mmol of the ligand (260.5 mg) was dissolved in 10 ml of methanol and a 5 ml methanolic solution of Ni(OAc)₂.6H₂O (1.5 mmol, 545 mg) was added to it with constant stirring. It was refluxed for 1 hour. The bottle-green solution was cooled to room temperature and then a methanolic solution of NaSCN (2 mmol, 162 mg) was added to it dropwise and very slowly. It was stirred for another one hour, filtered and kept overnight for slow evaporation. Deep-green coloured needle-shaped single crystals were obtained which were suitable for X-ray diffraction. Yield=580 mg (77%).

Elemental analysis calcd (%) for C_{31.50}H_{34.50}Cl₂N₂Ni₂O₂S₂: C 46.17, H 4.24, N 8.55, O 14.16, S 3.91, Cl 8.65, Ni 14.32; found C 46.21, H 4.33, N 8.49, O 14.10, S 3.85, Cl 8.60, Ni 14.42. ESI-MS m/z: Calcd for [C_{29}H_{24}N_{5}O_{2}Cl_{2}S_{3}Ni_{2}]^+ = 694.895, Found=693.938; FT-
IR data (KBr pellet): γ_{bar}= 2092 (SCN), 1642 (C=N), 1585 (benzene ring skeletal), 1469 (acetate bridge) cm\(^{-1}\).

**X-ray crystallography.** The crystal structure analysis of complex 1 was carried out at room temperature on a Bruker Smart Apex diffractometer equipped with CCD and Mo-K\( \alpha \) radiation (\( \lambda = 0.71073 \) Å). Cell refinement, indexing, and scaling of the data set were done by using the Bruker Smart Apex and Bruker Saint packages. The structure was solved by direct methods and subsequent Fourier analyses and refined by the full-matrix least-squares method based on \( F^2 \) with all observed reflections. The difference Fourier map showed an unreliable high thermal motion for the methyl group of methanol bound at Ni1, and the ligand was interpreted as a mixed species of water and methanol of 0.5 occupancy each. The packing show channels along axis c filled by disordered water molecules: one molecule disordered over three positions with occupancies 0.4/0.3/0.3 and an additional one with occupancy 0.25, of which H atoms not located. All the calculations were performed using the WinGX System, Ver2013.2. Crystal data and details of refinements are given in Table 6B.1.

**Table 6B.1. Crystallographic data and Details of Refinement for Ni\( ^{II} \) Complex.**

<table>
<thead>
<tr>
<th>empirical formula</th>
<th>C(<em>{31.50})H(</em>{34.50})Cl(_2)N(_3)Ni(<em>2)O(</em>{7.25})S</th>
</tr>
</thead>
<tbody>
<tr>
<td>formula weight</td>
<td>819.52</td>
</tr>
<tr>
<td>crystalsystem</td>
<td>Monoclinic</td>
</tr>
<tr>
<td>spacegroup</td>
<td>(P2_1/c) (No. 14)</td>
</tr>
<tr>
<td>a (Å)</td>
<td>18.463(2)</td>
</tr>
<tr>
<td>b (Å)</td>
<td>13.6212(18)</td>
</tr>
<tr>
<td>c (Å)</td>
<td>15.5388(18)</td>
</tr>
<tr>
<td>β (°)</td>
<td>92.291(13)</td>
</tr>
<tr>
<td>Volume (Å(^3)</td>
<td>3904.8(8)</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
</tr>
</tbody>
</table>
Detection of Hydrogen Peroxide in the Catalytic Reaction involving Catecholase Activity. The formation of H$_2$O$_2$ during the catalytic reaction was detected by following the development of the characteristic band for I$_3^-$ spectrophotometrically ($\lambda_{\text{max}} = 353$ nm; $\varepsilon = 26000$ M$^{-1}$ cm$^{-1}$), upon reaction with I$^-$. The formation of I$_3^-$ could be monitored spectrophotometrically because of the development of the characteristic I$_3^-$ band ($\lambda_{\text{max}} = 353$ nm; $\varepsilon = 26000$ M$^{-1}$ cm$^{-1}$).

Phosphatase activity: Kinetic Measurements of the Hydrolysis of 4-NPP in DMF. The same procedure was followed as described in Chapter 2A.

Biological activity.

Culture media and chemicals. Histopaque 1077 and Rhodamine B were procured from Sigma, RPMI 1640, penicillin, streptomycin, doxorubicin were procured from Sigma (St.

<table>
<thead>
<tr>
<th>Calc density (g/cm$^3$)</th>
<th>1.394</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu$ (Mo-K\textalpha) [mm]</td>
<td>1.203</td>
</tr>
<tr>
<td>F(000)</td>
<td>1690</td>
</tr>
<tr>
<td>Theta Min-Max [Deg]</td>
<td>1.86-24.11</td>
</tr>
<tr>
<td>Collected reflections</td>
<td>18366</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>6098</td>
</tr>
<tr>
<td>$R_{\text{int}}$</td>
<td>0.082</td>
</tr>
<tr>
<td>Observed reflections [I&gt;2$\sigma$(I)]</td>
<td>3025</td>
</tr>
<tr>
<td>Parameters</td>
<td>451</td>
</tr>
<tr>
<td>Goodness-of-fit on $F^2$</td>
<td>0.920</td>
</tr>
<tr>
<td>$R_1$, $wR2$</td>
<td>0.0767, 0.1766</td>
</tr>
<tr>
<td>residuals (e Å$^{-3}$)</td>
<td>0.609, -0.619</td>
</tr>
</tbody>
</table>
CHAPTER 6B

Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GIBCO/Invitrogen. Sodium chloride (NaCl), sodium carbonate (Na$_2$CO$_3$), sucrose, Hanks balanced salt solution (HBSS), HEPES–Na$^+$ buffer, 3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Himedia, India., Tris–HCl, Tris buffer, KH$_2$PO$_4$, K$_2$HPO$_4$, HCl, formaldehyde, alcohol, Titron X-100, Sodium dodecyl sulphate (SDS), phenol, chloroform, iso-amyl alcohol, ethidium bromide (EtBr), 2-vinylpyridine and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. Commercially available dimethyl sulfoxide (DMSO) was procured from Hi-media, India, and was purified by vacuum distillation over KOH. All the microbiological media and chemicals were obtained from HiMedia Laboratories, India. Ultrapure Milli Q water was used throughout the study.

**Cell lines and bacteria culture and maintenance.** KG-1A and K562 cell lines were obtained from NCCS, Pune (India). All cell lines were cultivated and maintained in RPMI-1640 complete media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U ml$^{-1}$ penicillin, 100 μg mL$^{-1}$ streptomycin and 4 mM L-glutamine under 5% CO2 and 95% humidified atmosphere at 37°C in CO2 incubator. Cells were cultured and maintained in logarithmic growth phase until number of cells reaches at 1.0 × 10$^6$ cells ml$^{-1}$. *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) bacteria were previously isolated in our laboratory. $^{8,9}$ The strains (MC 4 – *E. coli* and MMC 20 – *S. aureus*) are all multi drug resistant strains and were subculture and used throughout the study.

**Drug preparation.** A 10 mg/ml stock of Complex was prepared by dissolving 10 mg of complex in DMSO. It was then serially diluted with RPMI media to prepare working concentrations. The amount of DMSO for each concentration, was never exceed >0.75%.

**Anti-cancer efficacy study.**

**Experimental design.** The same procedure was followed as described in Chapter 2B.

**In vitro cell proliferation assay.** The same procedure was followed as described in Chapter 2B.

**In vitro drug uptake Assay.** The same procedure was followed as described in Chapter 2B.

**Intracellular ROS measurement.** The same procedure was followed as described in Chapter 2B.
**Pretreatment with N-acetyl-L-cysteine.** To determine the role of ROS in complex induced cell death, KG-1A and K562 cells were seeded in a 96-well plate at 0.2 mL per well at a concentration of $2 \times 10^5$ cells per milliliter. A stock solution of N-acetyl-L-cysteine (NAC; Sigma-Aldrich) was made with sterile water and added to cells at 5 and 10 mM for 1 h. After NAC pretreatment, cells were cultured with complex at IC50 dose for 24 h. Viability was determined by the MTT method.

**Measurement of mitochondrial membrane potential ($\Delta \Psi_m$).** The alteration of mitochondrial membrane potential by Spectro-fluorometric method was done according to previously reported method. Briefly, both the control and experimental cells exposed to our complex at IC$_{50}$ conc for 18 hr, were washed, suspended in ice-cold PBS. Approximately $1 \times 10^6$ cells ml$^{-1}$ no. of cells (KG-1A and K562) were incubated with 10 mM Rh 123 at 37ºC for 30 min and then washed twice with PBS. The cellular fluorescence intensity of Rh 123 was monitored for 2 min using Hitachi F-7000 Fluorescence Spectrophotometer. The cellular mitochondrial membrane potential was expressed as a percentage of control cells at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Both excitation and emission slit width were set to 5.0. All measurements were done in triplicate.

**DNA fragmentation Assay.** The same procedure was followed as described in Chapter 2B.

**Cellular morphology analysis by Acridine orange (AO)–ethidium bromide (Et Br) double staining.** To confirm the probable pathway of cell death we analyzed the cells by EtBr-AO double staining method. A number of $2 \times 10^4$ KG-1A and K562 cells were seeded into each well of a 6-well plate and incubated for 24 h at 37 ºC in a humidified, 5% CO$_2$ atmosphere. Complex at IC$_{50}$ dose then added into the well for 24 hr. After incubation, cells were washed once with phosphate buffer saline (PBS). Ten microlitres of the cells were then put on a glass slide and mixed with 10μl of acridine orange (50μg/ml) and ethidium bromide (50μg/ml). The cells were viewed under a fluorescence microscope (NIKON ECLIPSE LV100POL) with 400X magnification.

**In vitro antibacterial activity.**

**Agar well diffusion.** Antibacterial activity of complex was studied by the agar-well-diffusion method, a bacterial suspension was layered onto sterile nutrient agar at 37ºC
using Petri dish (diameter, 90 mm) on a horizontally leveled surface. After the medium had dried, 6-mm-diameter wells were made in the agar (at six wells per dish) that were equidistant from one another and from the dish edge. The wells received 20 μl of only DMSO solution and 25 μg, 50 μg, 100 μg, 200 μg and 400 μg complex dissolved in DMSO solution. The Petri dishes were incubated in a thermostat at 37ºC for 24 h. After incubation, the diameter of the zone of bacterial-growth inhibition was measured with an accuracy of ±0.1 mm. The mean inhibition-zone diameter and the maximal data scatter also were determined. All experiments were repeated thrice.39

**Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of complex.** The MIC and MBC of the complex were determined according to the method of Vijayan et al. with some modification. Twenty microlitres (~1 × 10^8 cells mL⁻¹) of each bacterial strains were added individually to 1 mL of nutrient broth (NB). The different concentrations (0, 5, 10, 25, 50, 100, 200, 400 and 800 μg/ml) of the complex were added to the test tubes containing the test strains. After 24 h of incubation, the MIC results were noted by checking the turbidity of the bacterial growth. To avoid the possibility of misinterpretations due to the turbidity of insoluble compounds, the minimum bactericidal concentration (MBC) was determined by sub culturing the MIC dilutions onto the sterile nutrient agar plates incubated at 37ºC for 24h. The lowest concentration of the complex which completely killed the tested bacteria was observed and tabulated as MBC level.

**Statistical analysis.** All the parameters were repeated at least three times. The data were expressed as mean ± SEM, n = 06. Comparisons between the means of control and treated group were made by one-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with multiple comparison tests, p<0.05 as a limit of significance.

**6B.3. Results and Discussion**

**Synthesis and Characterisation.** The Schiff-base ligand and the complex was synthesised and characterized by the conventional method. In FTIR spectrum, the small
separation in the two IR vibrations is indicative of the bidentate mode for the carboxylate group (1462 cm$^{-1}$ and 1432 cm$^{-1}$). Finally we find a sharp peak for methanolic O-H bond stretching at 2924 cm$^{-1}$ (Figure 6B.1-6B.2).

**Figure 6B.1.** FT-IR spectrum of ligand HL at 298 K.

**Figure 6B.2.** FT-IR spectrum of complex at 298 K.

**UV-vis spectra.** The absorption spectra of the ligand features three to five absorption bands where the band at 227-268 nm is assigned to $\pi\rightarrow\pi^*$ and that around 320-430 nm is probably for n→$\pi^*$ transition (Figure 6B.3A). The solid state UV-vis spectra indicates three absorption bands at 600, 916 and 1190 nm for d-d transition and another highly intense band at 367 nm for LMCT transition which corroborates well with an octahedral
structure (Figure 6B.4).\textsuperscript{42,43} Solution electronic spectral diagram shows a highly intense band at 231-270 nm and a moderately intense band at \(\sim\)375 nm in both acetonitrile and DMF solvent which can be assigned to ligand-to-metal charge transfer transitions (Figure 6B.3B).\textsuperscript{44}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6b_3}
\caption{Absorption spectra of (A) free ligand HL in Acetonitrile and DMF medium of 10\textsuperscript{-4} (M) solution. Inset shows the same in 10\textsuperscript{-2} (M) solution (B) complex in solid state at 298 K.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6b_4}
\caption{Absorption spectra of complex in acetonitrile and DMF medium of a solution of concentration (A) 10\textsuperscript{-4} M (B) 10\textsuperscript{-2} M at 298K.}
\end{figure}
**NMR studies.** The ligand has been characterised by both $^1$H and $^{13}$C NMR spectral analysis. In proton NMR, the Schiff-base hydrogen is found to resonate at δ 8.127 as a singlet and there are two triplets in the region δ 3.072-3.982 for the methylene protons of N-CH$_2$-CH$_2$ group. The aromatic protons are found to resonate in the region δ 6.774-8.490 for both benzene and pyridine ring and that for phenolic-OH at 13.3 as a broad singlet (Figure 6B.5-6B.6).

![Figure 6B.5. $^1$H NMR spectrum of ligand HL in CDCl$_3$ at 298K.](image)

**Figure 6B.6. $^{13}$C NMR spectrum of ligand HL in CDCl$_3$ at 298K.**

**Thermogravimetric analysis.** Thermal study of the complex in the temperature range 30-700°C shows well-defined step-wise decomposition (Figure 6B.7) upon heating. It is
thermally stable up to 49 °C. The thermogram represents two consecutive steps where the weight loss in the first step is 9.91% (calcd. 9.82%) corresponding to the elimination of 1.25 molecules of water of crystallisation (attached to Ni1) and the thiocyanate group (attached to Ni2) and in the second step is 75.42% (calcd. 79.83%) for the rest part of the molecule leading to nickel sulphide and nickel oxide as the end product.

**Figure 6B.7. TGA curve of complex from 30 °C – 700 °C.**

**Structure description.** The X-ray structural determination of the complex reveals a discrete neutral dinuclear nickel(II) complex. An ORTEP view of the complex with an atom labeling scheme is shown in Figure 6B.8A, crystal data refinements in Table 6B.1 and a selection of bond lengths and angles is reported in Table 6B.2.

Each metal atom is chelated by a Schiff base ligand through the imino and pyridine nitrogen donors and by the phenoxo oxygen that in turn acts as bridging species connecting the other metal. In addition the metals are bridged by an acetate anion so that the metals are separated by 3.099(1) Å. The chromophor arrangement in each coordination sphere is different, being N2O4 for Ni1 and N3O3 for Ni2 so that assuming the equatorial plane of Ni(II) ions formed by the tridentate ligand and the acetato oxygen, the axial positions are occupied by the phenoxo oxygen of the other ligand and by the thiocyanate nitrogen (N5) at Ni2 and the acqua oxygen (O3) at Ni1 to complete their distorted octahedral geometry. It is
worth noting that the tridentate ligands are arranged in head-tail fashion and the angle formed by the mean planes through the hexa-nuclear coordination cycles of the chelating Schiff base N,O donors is of 32.5° (Figure 6B.8B).

The Ni-N(imine) bond distances are comparable in length [2.067(6), 2.081(6) Å] and both are slightly shorter than Ni-N(py) ones of 2.132(6) and 2.137(7) Å, for Ni1 and N2, respectively. On the other hand the Ni-NCS distance is considerably shorter being of 2.054(6) Å. The Ni-O(phenoxo) and Ni-O(acetate) bond lengths appear comparable falling in a range from 2.059(5) to 2.076(5) Å, significantly shorter with respect the value measured for disordered methanol/aqua ligand of 2.166(6) Å.

![Figure 6B.8. (A) ORTEP view of the dinuclear complex. (ellipsoid probability at 30%). The water molecule O3 shares the coordination site with a methanol molecule (not shown). (B) Side view of the complex with indication of the dihedral angle between the mean planes through the hexa-nuclear coordination cycles formed by the chelating Schiff base N,O donors.](image)

**Table 6B.2.** Selected Coordination Bond Lengths (Å) and Angles (deg) for 1 with Esds in Parentheses.

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length (Å)</th>
<th>Bond</th>
<th>Length (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni(1)-O(1)</td>
<td>2.077(5)</td>
<td>Ni(2)-O(2)</td>
<td>2.061(4)</td>
</tr>
<tr>
<td>Ni(1)-N(1)</td>
<td>2.069(6)</td>
<td>Ni(2)-N(3)</td>
<td>2.080(6)</td>
</tr>
<tr>
<td>Ni(1)-N(2)</td>
<td>2.135(6)</td>
<td>Ni(2)-N(4)</td>
<td>2.138(7)</td>
</tr>
<tr>
<td>Ni(1)-O(2)</td>
<td>2.105(4)</td>
<td>Ni(2)-O(1)</td>
<td>2.197(4)</td>
</tr>
<tr>
<td>Ni(1)-O(3)</td>
<td>2.163(6)</td>
<td>Ni(2)-O(4)</td>
<td>2.078(5)</td>
</tr>
<tr>
<td>Ni(1)-O(5)</td>
<td>2.056(5)</td>
<td>Ni(2)-N(5)</td>
<td>2.054(6)</td>
</tr>
<tr>
<td>Bond Description</td>
<td>Angles (°)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(1)-Ni(1)-N(1)</td>
<td>89.9(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(1)-Ni(1)-N(2)</td>
<td>93.1(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(1)-Ni(1)-N(2)</td>
<td>176.0(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(1)-Ni(1)-O(2)</td>
<td>84.89(17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(1)-Ni(1)-O(2)</td>
<td>93.3(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(2)-Ni(1)-N(2)</td>
<td>97.50(19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(5)-Ni(1)-O(1)</td>
<td>87.94(19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(5)-Ni(1)-N(1)</td>
<td>177.2(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(5)-Ni(1)-N(2)</td>
<td>89.0(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(1)-Ni(1)-O(3)</td>
<td>88.1(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(1)-Ni(1)-O(3)</td>
<td>94.2(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(2)-Ni(1)-O(3)</td>
<td>89.1(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(5)-Ni(1)-O(2)</td>
<td>88.32(18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(5)-Ni(1)-O(3)</td>
<td>83.9(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(2)-Ni(1)-O(3)</td>
<td>169.7(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ni(1)-O(1)-Ni(2)</td>
<td>92.88(17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(2)-Ni(2)-N(3)</td>
<td>91.6(3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(2)-Ni(2)-N(4)</td>
<td>173.7(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(3)-Ni(2)-O(2)</td>
<td>82.96(17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(3)-Ni(2)-O(1)</td>
<td>93.04(19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-O(1)</td>
<td>91.1(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-N(4)</td>
<td>88.02(19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-N(3)</td>
<td>178.5(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-N(4)</td>
<td>89.6(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-N(3)</td>
<td>93.5(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-O(1)</td>
<td>90.9(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-N(4)</td>
<td>93.3(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-O(1)</td>
<td>87.64(18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-N(3)</td>
<td>88.3(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-N(4)</td>
<td>174.1(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O(4)-Ni(2)-O(1)</td>
<td>96.13(18)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Solution structure.** Electrospray ionisation data in the positive mode shows the base peak at 693.938 m/z value (Figure 6B.9).

![Figure 6B.9. ESI-MS of complex in Acetonitrile. Inset shows the solution structure.](image-url)
Molar conductance studies show that the complex behaves as an 1:1 electrolyte in solution (151 μS in Acetonitrile and 70 μS in DMF). Thus the results indicate that it dissociates into a monopositive species where the acetate bridge and methanolic group dissociates into the solution (Figure 6B.12).

**Catecholase activity.** The catecholase activity of the Ni\(^{II}\)-complex has been investigated in two solvents: Acetonitrile and DMF employing the two model substrates, DTBC and TCC, following the protocol adopted in biomimetic studies. The oxidation product 3,5-di-tert-butylquinone (3,5-DTBQ) is highly stable and shows a maximum absorption at around 400 nm (Figure 6B.10(i)). The kinetic plots has been shown in Figure 6B.10(ii).

![Figure 6B.10(i). Wavelength scan of a mixture of complex and DTBC in the molar ratio 1:100 in acetonitrile solvent for 1 hour at an interval of 5 minutes. Conditions: \([DTBC]=10^{-2} \text{ (M)}, [\text{complex}]=10^{-4} \text{ (M)}, \text{Temperature}=298K.\)]
Figure 6B.10(ii). Kinetics plot (rate versus [DTBC]) of the catecholase activity of complex in (A) Acetonitrile with excess substrate conditions. Inset shows the Line-weaver Burk plot of the same ($R^2=0.978$, Standard deviation=1.7, Slope=0.02118 sec, Error=0.00202, Intercept=10.976 M$^{-1}$sec, Error=0.832) (B) DMF with excess substrate conditions. Inset shows the Line-weaver Burk plot of the same ($R^2=0.976$, Standard deviation=0.163, Slope=0.00196sec, Error=0.000194, Intercept=3.567 M$^{-1}$sec, Error=0.0798)

With TCC, we observe that an adduct is formed in acetonitrile which breaks down gradually with time (Figure 6B.11A) while the same happens in DMF which does not change with time (Figure 6B.11B). The control experiments prove the non-participation of the ligands in Figure 6B.12.
Figure 6B.11. Wavelength scan of catecholase activity of complex with tetrachlorocatechol (TCC) in (A) Acetonitrile and (B) DMF. Conditions: \([TCC]= 10^{-2} \) (M), \([\text{Complex}]= 10^{-4} \) (M), Temperature=298 K.

Figure 6B.12. Control experiment for catecholase activity in Acetonitrile and DMF with ligand and 3,5-di-tert-butylcatechol (DTBC) where spectra is recorded after 1 hour of mixing. Conditions: \([\text{DTBC}]= 10^{-2} \) (M), \([\text{HL}]= 10^{-4} \) (M), Temperature=298 K.

**ESI-MS studies.** During catecholase activity, a 1:1 adduct is formed between catecholate anion and metallocatalyst which is displayed as the base peak at m/z 855.9 ([C_{42}H_{45}Ni_{2}O_{4}Cl_{2}]+; calculated m/z=857) in acetonitrile as shown in Figure 6B.13.
Cyclic voltammetric studies. In cyclic voltammetric experiments, on the oxidative side, in MeCN solution, four oxidative responses are obtained at 0.75, 1.1, 1.3 and 1.6 V (Figure 6B.14). All these four electrode processes are irreversible, with the corresponding reductive waves being barely observable. The first oxidative response is tentatively assigned to Ni(II)/Ni(III) oxidation, while the second oxidative response is probably due to oxidation of thiocyanate. At more positive potentials oxidation of phenoxy ions to phenoxy radicals are observed. On the reductive side a broad irreversible reductive wave at \(-1.57\) V (Epc = \(-1.57\) V, Epa = \(-1.12\) V) is assigned to azomethine reduction. In DMF solution, only two irreversible broad oxidative waves at 0.9 and 1.45 V, are observed in cyclic voltammetry, while in differential pulse voltammetry two broad peaks of relatively equal height, at 0.74 V and 1.21 V followed by a shoulder at 1.43 V are observed (Figure 6B.15). The broad oxidation at 0.9 V is probably due to overlapping oxidations of Ni(II) and thiocyanate, while at higher positive potentials oxidations of phenoxy ligands are observed. On the reductive side, in DMF solution, a quasi-reversible wave at \(-1.3\) V (Epc = \(-1.48\) V, Epa = \(-1.13\) V ∆Ep = 350 mV) is observed.

There is a second reductive peak at \(-1.7\) V, which overlaps with the first reductive peak, but it is more clearly discernable in the differential pulse voltammogram (Figure 6B.15). The reductive process at \(-1.7\) V may be due to Ni(II)/Ni(I) reduction or it may also be due
to addition of electron to the $\pi^*$ orbital of pyridine. The lower azomethine reduction potential in DMF solution, along with the more reversible nature of this redox couple may explain the better catecholase activity of the compound in DMF solution.

**Mechanistic pathways.** The mass-spectrometry data, corroborated by electron paramagnetic resonance (EPR) measurements (Figure 6B.16A), suggest that the metal centers are not directly involved in the catecholase activity but it is a metal-mediated ligand-centred radical catalytic pathway which is exhibited by the complexes. The liberation of $\text{H}_2\text{O}_2$ in the last step of the cycle is detected spectrophotometrically by the generation of $\text{I}_3^-$ band (Figure 6B.16B). Since the stability of the intermediate C is very vital in the cycle, the oxidation will be facilitated in presence of a more polar solvent as explained from Figure 6B.17.
Figure 6B.16. (A) EPR spectra of a mixture of complex and DTBC (1:100 molar stoichiometry) (B) Absorption spectra of $I_3^-$ for $H_2O_2$ detection in catecholase activity at 298K.

Figure 6B.17. Proposed mechanistic pathway involved in the catalytic cycle of catecholase activity in Acetonitrile medium.

**Phosphatase activity.** The Phosphatase activity of our complex was investigated employing the substrate disodium (4-nitrophenyl)phosphate hexahydrate and the study was performed in both Acetonitrile and DMF. While the complex showed no activity in acetonitrile, it is a quite good catalyst for phosphomonoester cleavage in DMF medium. The hydrolytic tendency of the complex was detected spectrophotometrically by monitoring the time evolution of $p$-nitrophenolate ($\lambda_{max} = 427$ nm) through wavelength scan from 200 to 800 nm in aqueous DMF where substrate was in 20 equivalents of the catalyst, till roughly 2% reaction conversion. The change in spectral behaviour of the
complex upon addition of 4-NPP is shown in Figure 6B.18 where spectra was recorded for 2 hours at an interval of 5 min.

**Figure 6B.18.** Wavelength scan for a mixture of complex and 4–NPP in the molar ratio 1:20 in DMF medium for 2 hours at an interval of 5 minutes. Conditions: [4-NPP]=10^{-3} \text{(M)}, [complex]=0.05 \times 10^{-3} \text{(M)}, Temperature=298K.

**Kinetic Studies.**
**Figure 6B.19.** Control experiment of Phosphatase activity with free ligand HL.

*Conditions: [4-NPP]=1 × 10^{-3} (M), [HL]=5 × 10^{-5} (M), Temperature=298 K.*

The kinetic study was done by the initial slope method following the rate of increase in absorption of the band at ~400nm corresponding to rise in 4-nitrophenolate concentration.

To confirm the non-catalytic property of the ligand, control experiments were performed (Figure 6B.19).

**Table 6B.3.** Kinetic Rate Constants for the Catalytic Activities of Ni^{II} complex in Acetonitrile and DMF at 25 °C.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Acetonitrile</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catecholase</td>
<td>9.11×10^{-4}</td>
<td>2.8×10^{-3}</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>-</td>
<td>8.18</td>
</tr>
</tbody>
</table>

**ESI-MS studies.** Electro-spray ionisation studies in the positive mode were measured after 10 minutes of mixing of complex and substrate. Although a small peak is formed for the 1:1 adduct at m/z 1072.0084 (C_{56}H_{32}N_{6}O_{9}Cl_{2}S_{1}P_{1}Ni_{2}; calculated m/z=1072), the base peak is found at m/z 1448.06 (C_{42}H_{36}N_{7}O_{15}Cl_{2}S_{1}P_{2}Ni_{2}; calculated m/z=1450) for the 2:1 adduct formed between p-nitrophenylphosphate and metal-complex (Figure 6B.20). Thus the major signal indicates the predominant presence of the intermediate where substrate and complex are in the molar proportion 2:1.
**Figure 6B.20.** ESI-MS of a mixture of complex and 4-NPP in DMF.

**Mechanistic implications.** We hereby propose a mechanism originating from the classical lewis-acid activation mechanism of the metal ion catalyzed phosphate ester hydrolysis. Here Ni(II)-coordinated H₂O/OH⁻ is the sole nucleophile.⁴⁵ Thus initially the nickel core recognizes and binds the phosphate bond through coordinate linkage and electrostatic interaction. Then it activates and nucleophilically attacks the central phosphorus atom by a metal-bound external water/hydroxide ion generating a trigonal bipyramid phosphorus intermediate from where the P–O ester bond is finally cleaved.⁴⁶-⁴⁹ Other research work¹⁶-²⁰ on dinuclear model complexes have also shown hydrolysis proceeds via “hybrid” mechanism, in which the substrate coordinates to the Lewis acidic (Ln)Zn(OH) species, thereby undergoing both Lewis acid activation and intramolecular attack by the metal-bound OH⁻. Thus precisely, the cleavage of 4-NPP promoted by the complex may be triggered by the phosphomonoester coordination to two nickel centers in a bidentate bridging mode separately, followed by an attack of the deprotonated alcoholic hydroxyl to the phosphorus center, and finally leading to cleavage of the P-O bond. The molecule B with 2:1 adduct between substrate and complex is the most possible intermediate in the catalytic cycle (Figure 6B.21).
Figure 6B.21. Probable enzyme-substrate (A) 1:1 (B) 1:2 adducts in phosphate ester hydrolysis as promoted by Ni\textsuperscript{II} complex.

In vitro anti leukemic activity.

In vitro cell viability study. Cell viability study showed that Complex exposure was significantly (p<0.05) caused a reduction in cell viability in leukemic cell lines by dose dependent fashion. In KG-1A cell line the viability was significantly decreased by 53.82%, 65.38% and 70.38% at 25, 50 and 100 μg ml\textsuperscript{-1} doses; whereas in K562 cell line this metal complex was able to reduced cell viability by 67.97 %, 79.31% and 83.32% at 25, 50 and 100 μg ml\textsuperscript{-1} doses respectively (Figure 6B.22). The IC\textsubscript{50} dose of this complex was found 43.57 μg/ml and 24.62 μg/ml for KG-1A and K562 cells respectively.

Figure 6B.22. In vitro cell viability assay of complex treated KG-1A and K562 cell lines. Cells were treated with the complex for 24 h at 37 °C. Cell viability was measured by the MTT method as described in materials and methods. Values are expressed as mean ± SEM of three experiments; * and # indicates significant difference (p < 0.05) compared to the control group.
In our study the metal complex showed potent anti-leukemic effects in both cell lines which is supported by many researchers. So, further experiments were done using IC$_{50}$ doses only.

**Complex internalization.** It was observed that any bioactive material binding to the plasma membrane and intracellular uptake are essential condition for its exertion of cytotoxicity. Result showed that the complex was taken up by KG-1A and K562 cell lines in *in vitro* cultures (Figure 6B.23). From the fluorescence microscopic images it was revealed that fluorescence tagged complex were distributed throughout the cytoplasm, which indicated the successful internalization of complex in the cells. The internalization processes of complex may be due to phagocytosis, pinocytosis or endocytosis have all been well studied and seem to strongly depend on particle form, size and cell type used. Effective cellular uptake of any anti-leukemic drug is necessary for exertion of potent cytotoxic effects.

*Figure 6B.23. Intracellular localization of complex in KG-1A and K562 cell lines by fluorescence imaging. A required amount of cells was treated with Rhodamine B labeled metal complex for 6 h. Intracellular uptake was examined using fluorescence microscope. Here, 2A: KG-1A cells, 2B: K562 cells.*
**Cellular ROS level.** Cellular ROS level in KG-1A cell line was increased by 2.34 fold at IC_{50} dose of complex when compared to control. In the same line of treatment ROS level was also significantly (p<0.05) increased by 3.61 fold at IC_{50} dose of complex when compared to control (Figure 6B.24). Moreover 1 μM H_{2}O_{2} treatment for 24 hr (positive control) significantly elevated ROS level by 3.45 fold and 4.29 fold in KG-1A and K562 cells.

![Figure 6B.24. Effects of complex on ROS induction in KG-1A and K562 cell lines.](image)

**Figure 6B.24.** Effects of complex on ROS induction in KG-1A and K562 cell lines. DCF fluorescence intensity was expressed in term of ROS production. (A) Results represent the means of three separate experiments, and error bars represent the standard error of the mean. Values are expressed as mean ± SEM of three experiments; superscripts indicate significant difference (p < 0.05) compared to the control group. Intensity of control cells were set to 100. Data is represented as fold change as compare to control group. (B) Qualitative presentation of ROS generation by fluorescence microscopy. Here, (i): Untreated KG-1A cells, (ii): Complex treated KG-1A cells, (iii): Untreated K562 cells, (ii): Complex treated K562 cells.

Reactive oxygen species (ROS) are special types of bioactive molecules or ions which contain unpaired valence electrons in their outer shell that makes them highly active and therefore plays potential role in cell signaling, leading to oxidative cell damage.\textsuperscript{54}
Physiologically active cellular metabolism produces various intracellular enzymes like glutathione enzymes, superoxide dismutase; catalase etc. comprises the potency to neutralize the toxic effects of ROS produced in moderate amount. In the present study the complex may have some diverse effects on the cellular redox balance which thereby produced oxidative stress in leukemic cells. Massive amounts of oxidative stress deplete glutathione precursors and thereby becoming them insufficient in neutralizing the ROS generated in leukemic cells. To confirm the effective participation of ROS in complex induced leukemic cell death, we pre-treated the KG-1A and K562 cells with NAC. After these pre-treatments, complex exposure was done at their respective IC50 doses for 24 h, followed by experiments where cell viability was estimated using the MTT method. We found that pre-treatment with NAC (ROS scavenger) protected the cells from the cytotoxic effects of complex. In this cases>90% of cell viability suggested that the complex selectively killed the leukemic cells by generation of ROS (Figure 6B.25), which activates several downstream signaling pathway leading to cell death by apoptosis.

![Figure 6B.25. Quenching of ROS rescues KG-1A and K562 cells from complex induced cytotoxicity. KG-1A and K562 cells were pre-treated with 5 mM and 10 mM N-acetyl cysteine (NAC) for 4-6 hrs and then subsequently exposed to Ag NPs at IC50 dose. Cell viability was estimated by MTT assay.](image)
**Alterations of mitochondrial membrane potential (MMP).** In our study mitochondrial membrane potential (MMP) was estimated in terms of rhodamin 123 fluorescence intensity. Mitochondria are known to be involved in the process of programmed cell death. Rh 123 is preferentially accumulates in the mitochondria of living cells and functions as a fluorescent chemical probe for mitochondria trans membrane potential. Results showed that the percentage of MMP decreased significantly (P<0.05) in KG-1A and K562 cell line by 41.37% and 48.85% respectively when treated with IC50 doses (Figure 6B.26). Decrease of MMP in complex treated leukemic cells may be due to malfunction in ATP synthesis and maintenance of ATP level that leading to either apoptosis or necrosis. Cellular apoptosis does not depend only depleted ATP synthesis, where as certain drop of ATP level helps apoptosis, induced cell death. In the time of apoptosis, nuclear disintegration can be preceded with loss or entire dissipation of the ΔΨm , which may occur shortly before or simultaneously with Bax translocation to mitochondria membranes.

**DNA fragmentation assay.** DNA cleavage activity of the complex was examined in both leukemic cell lines. It was noted that the complex showed potent DNA fragmentation activity in both systems. In KG-1A and K562 cells, DNA fragmentation was increased by 238.83 % and 441.55% respectively at IC50 dose of the complex (Figure 6B.27). DNA damage is one of the strong evidence of apoptosis activity. In our study, Ni complex induced DNA damage is highly in accordance with the previous study of many researchers. The DNA fragmentation by the complex may be caused due to activation of endogenous nuclear endonuclease, which selectively and distinctively cleaves the double-stranded nuclear DNA. In our study, elevated level of ROS may contribute to severe genotoxic effects in leukemic cell lines.
Figure 6B.26. (A) Measurement of mitochondrial membrane potential (MMP) of complex treated KG-1A and K562 cell lines. Mitochondrial membrane potential was estimated by measuring Rhodamin 123 fluorescence intensity. Data is represented as the percentage of the MMP level in the control group. Values are expressed as mean ± SEM of three experiments; * and # indicates significant difference (p < 0.05) compared to the control group. (B) Quantitative estimation of DNA fragmentation assay by diphenylamine (DPA) assay. Values were expressed as Mean ± SEM; n=03, * and # indicates significant difference (p < 0.05) compared to the control group.

**Et Br-AO double staining cell morphological analysis.** This staining reveals that the viable cells with intact DNA and nucleus give a round and green nuclei. Early apoptotic cells will have fragmented DNA which gives several green colored nuclei. Late apoptotic and necrotic cell’s DNA would be fragmented and stained orange and red. From the images it was found that the complex was able to decrease the number of viable cells tremendously. Most of the cells exhibited typical characteristics of apoptotic cells like plasma membrane blebbing and formation of apoptotic bodies. The significant number of leukemic cells stained orange was increase. A very small amount of cells were stained as red colour. These indicated that most of the leukemic cells were not undergoing necrosis rather cell death occurred primarily through apoptosis. The results obtained from the Et Br-AO double staining are shown in Figure 6B.28.
In vitro antibacterial activity

Disc agar diffusion. The antibacterial activity of complex was tested on gram negative \textit{Escherichia coli} (\textit{E. coli}) and gram positive \textit{Staphylococcus aureus} (\textit{S. aureus}) bacteria using the diameter of inhibition zone in disk diffusion test. The diameter of inhibition zone (DIZ) is visual evidence which reflects susceptibility pattern of the microorganisms towards the exposed metal complex. The disks with complex were surrounded by a larger DIZ in \textit{S. aureus} and lower DIZ in \textit{E.coli} strains. DIZ of the complex on \textit{E.coli} was significantly (p<0.05) increased from 100 μg dose of complex and reached at 16.5 mm at 400 μg dose. In case of \textit{S. aureus}, the DIZ significantly (p<0.05) elevated from 50 μg dose of complex and ultimately reached at 20.5 mm at highest dose (Figure 6B.29A, B and C). It was noticed that paper soaked in complex with different concentrations had a significant inhibition zone against \textit{E. coli} and \textit{S. aureus}. The complex showed better effect on
S. aureus. This differential activity may be due to the structural difference in cell wall composition of Gram-positive and Gram-negative bacteria. Gram-positive bacteria possess a thick layer of peptidoglycan (20–80 nm). It is well known that Gram-negative bacteria possess an outer membrane outside the peptidoglycan layer lacking in Gram-positive organisms. The important role of the outer membrane is to serve as a selective permeability barrier to protect bacteria from harmful agents, making it difficult for the complex to penetrate.\(^5\)

**Figure 6B.28.** Anti-microbial sensitivity pattern of complex by disc agar diffusion method. Here, *A:* Graphical representation of diameter of inhibition zone. *B:* Disc agar diffusion pattern of multi drug resistant *E. coli* strain and *C:* Disc agar diffusion pattern of multi drug resistant *S. aureus* strain. All strains were exposed to 20 μl of only DMSO solution.
(a) and 25 μg (b), 50 μg (c), 100 μg (d), 200 μg (e) and 400 μg (f) complex dissolved in DMSO solution containing disc and subsequently incubated for 24 hr at 37°C. Inhibition zones were recorded by using zone scale. Values are expressed as mean ± SEM of three separate experiments; * and # indicates significant difference (p < 0.05) compared to the control group.

**Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of NPs.** The complex was observed to exhibit less antimicrobial activity on gram-negative microorganism than gram-positive ones.

**Figure 6B.29.** Determination of MIC value of complex for multi drug resistant E. coli strain. MIC of complex for MC 4 isolate was 400μg/ml. Here, -ve: negative control, + ve: positive control, a: 5 μg/ml, b: 10 μg/ml, c: 25 μg/ml, d: 50 μg/ml, e: 100 μg/ml, f: 200 μg/ml, g: 400 μg/ml. **27B:** Determination of MIC value of complex for multi drug resistant S. aureus strain. MIC of Ag NPs for
Particular drug concentration was noted where no visible growth appears in *S. aureus* and *E. coli* broth culture in case of complex exposure. MIC value for *S. aureus* strain, was 200 μg/ml, where as in case of *E. coli* strain, the MIC value was 400 μg/ml (Figure 6B.30). The MIC of samples is higher when tested against *E. coli*. The MBC values were 400 μg/ml and 800 μg/ml in *S.aureus* and *E.coli* strains respectively (Figure 6B.41). The growths are inhibited may due to the penetration of Ni ions into the bacterial cell that inhibits the bacterial growth and acts as a bactericidal agent followed by bacteriostatic activity. Here again the cell wall content plays an important role for those results.

**Figure 6B.30.** Determination of MBC value of complex for multi drug resistant *E. coli* strain. MBC of Ag NPs for MC 4 isolate was 800 μg/ml. Here, -ve : negative control, + ve : positive control, a : 5 μg/ml, b : 10 μg/ml, c: 25 μg/ml, d : 50 μg/ml, e: 100 μg/ml, f: 200 μg/ml, g: 400 μg/ml, h : 800 μg/ml.

**Figure 6B.41.** Determination of MBC value of complex for multi drug resistant *S. aureus* strain. MBC of Ag NPs for MMC 20 isolate was 400 μg/ml. Here, -ve : negative control, + ve : positive control, a : 5 μg/ml, b : 10 μg/ml, c: 25 μg/ml, d : 50 μg/ml, e: 100 μg/ml, f: 200 μg/ml, g: 400 μg/ml, h : 800 μg/ml.

### 6B.4. Conclusion

The present work derives deep-rooted impetus from the active sites of metallohydrolases and catecholase model systems which simultaneously possess biological efficacy. As a
continuation of our activity on bioinspired coordination chemistry with phenoxo-/hydroxo-bridged dinickel(II) complexes, we wished to synthesize phenoxo-bridged dinickel(II) complex with labile site(s) to estimate its versatality in catalysis cum biochemistry and to evaluate its progressiveness in comparison to previously synthesized complexes. Thereby, a novel dinuclear Ni(II) complex has been synthesised and characterised by routine physicochemical techniques and X-ray single crystal structure analysis. The results addressed herein reveals that it has high potency towards hydrolysis of organic phosphate ester and in mimicking catecholase activity. It is also reasonable to infer that potent anti-proliferative activity has been shown against KG-1A and K562 cell lines out of which better results have been obtained in case of latter. Elevation of ROS and disruption of mitochondrial membrane potential in complex exposed leukemic cells suggested the possible contribution of apoptosis underlying the etiology of cell death. The involvement of apoptosis rather than necrosis was confirmed by EtBr-AO double staining method. The antimicrobial activity of the complex was quite promising, suggests the further use of the complex in bio-medical applications. However in vivo study is necessary to enlighten the effect of complex in system level.

6B.5. References