Pharmacological approach for bio-relevant N-functionalized homo-binuclear macrocyclic complexes based on 16-membered tetraaza units: Synthesis, spectral studies, biological screening, HSA binding and molecular docking
5.1 INTRODUCTION

During the past few years macrocyclic complexes and their coordination chemistry have been emerging as a thrust area of current research for their architectural diversity with multinuclear metals having several applications such as in the catalysis, photosynthesis and dioxygen transport, metalloenzymes, metal extractants and radio therapeutic agents. There are several reports that investigated various macrocycles both natural and synthetic, thereby exploring the unusual coordination properties of cyclic ligand complexes. The cyclic arrangement resulted from the huge number of donor atoms and the flexibility of these ligands facilitate their role as a good host for ions. Hence, they find ample applications in supramolecular chemistry to mimic some biological molecules like metalloenzymes. When transition metal ion coupled with bicompartamental scaffolds, the pharmacological efficacy is enhanced manifold which display independent or cooperative behavior depending upon bridge length, linking the two subunits. There have been many attempts for the synthesis of cyclam-based homo/hetero dinuclear complexes by different research groups. Recently, Sandhanamalar et al. reported different macrocyclic complexes containing 3, 3'-diaminobenzidine moiety as bridging molecule. The binuclear complexes of Cu(II) have garnered large interest to researchers because of their biocompatibility, structural stability and variability. The binuclear Schiff base complexes derivatives of 3,3'-diaminobenzidine have displayed pronounced DNA cleavage, DNA binding, SOD and antimicrobial potencies. In another study, human serum albumin (HSA) being the suitable candidate for drug delivery, is the most widely studied multifunctional transport protein as it exhibit decreased immunogenicity. Most of the drugs circulate in blood plasma which ultimately reached to target tissues upon binding to HSA, because HSA controls drugs distribution. HSA has many essential physiological functions such as in the maintenance of the colloid osmotic pressure in blood and transportation, distribution, deposition of several exogenous and endogenous substances to their specified molecular target. A distinctive drug efficacy is observed in case of high binding affinity while low binding affinity results in poor dispersion and short life-span in blood. Thus, HSA binding efficiency of drugs is an important aspect to be considered in order to determine its pharmacokinetics e.g., prolonging in-vivo half-life, restricts the unbound concentration and thus cause drug distribution and elimination. In addition, HSA accumulates in tumor and act as carrier conjugate for
a variety of anticancer drugs viz., doxorubicin, chlorambucil and paclitaxel.\textsuperscript{16} In comparison to the studies based on the binding properties of mono-nuclear macrocyclic complexes, there is relatively less number of studies on the elucidation of HSA binding in case of homo-binuclear macrocyclic complexes. Therefore, understanding the mechanism of drug interaction with serum albumin is of great interest. In addition, diseases caused by bacterial infection are emerging as serious health concern worldwide affecting both economical and social sectors. Rise in bacterial antibiotic resistance and mutations along with hospital associated infections are the global hazards to all the agents. For instance, infections due to \textit{Shigella flexneri} results in annual death of 1.5 million people, since it contaminate foods and drinks.\textsuperscript{17} Therefore, the development of novel antibacterial agents against bacteria strains mostly major food pathogens, such as \textit{S. aureus}, \textit{P. aeruginosa} etc. has become an utmost requirement. The present work is intended to explore these problems to induce further investigations by addressing new techniques, benefiting from the distinctive properties of macrocyclic ligands. In view of the fact, antioxidants are natural or synthetic compounds that minimize the free radicals adverse effects. The behaviour of new metal complexes as an antioxidant species has been emerging as a focused area of interest in recent years. There are many scientific reports that suggest that the risk of several chronic diseases such as heart and cancer maladies,\textsuperscript{18,19} can be minimized with the help of antioxidants.

In the present study, the tetraaza bis(macro cyclic)-bimetal(II) complexes, [\(M_2LCl_4\)].\(nH_2O\), synthesized by metal ion controlled reaction between 3,3'-diaminobenzidine with 1,2-bis(bromomethyl)benzene and ethane-1,2-diamine intended to explore the \textit{in-vitro} antibacterial, biofilm inhibition, antioxidant properties and HSA binding studies.

\section*{5.2 EXPERIMENTAL}

\subsection*{5.2.1 Materials and methods}
All reagents were of analytical grade and used as received without prior purification. The metal salts and 3,3'-diaminobenzidine, 1,2-bis(bromomethyl)benzene, ethane-1,2-diamine, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), phosphate buffer saline (PBS), human serum albumin (A1887), menadione and 2,2-diphenyl-1-picryl hydrazyl (DPPH) were procured from Sigma-Aldrich.
Culture media Luria agar and Luria broth were obtained from Hi-media (India). Bacterial strains were procured from Microbial Type Culture Collection (MTCC), (IMTECH) (India).

5.2.2 Physical measurements
Elemental analysis (C, H and N) was obtained using Perkin Elmer elemental analyzer. The FTIR spectra (prepared as KBr pellets) were recorded on a Perkin Elmer-2400 spectrometer in the range of 4000-400 cm\(^{-1}\). The NMR spectra (\(^1\)H and \(^{13}\)C) were measured in appropriate solvent (DMSO-d\(_6\)) using TMS as internal standard on Bruker Avance II 400 NMR spectrometer. The mass spectra were obtained using a WATERS Q-TOF premier mass spectrometer. The electronic spectra in DMSO (10\(^{-3}\)) were obtained by UV-vis spectrometer Lambda-25 with quartz cuvettes (path length = 1 cm) while the conductivity measurements were carried out on freshly prepared (10\(^{-3}\) M) in DMSO solutions at room temperature on a Systronic type 302 conductivity bridge equilibrated at 25±0.01 °C. Magnetic susceptibility measurement was done at room temperature using magnetic susceptibility balance, Sherwood scientific Cambridge U.K. The EPR spectrum of Cu(II) complex was quantified using DPPH as standard (g = 2.0036) at 9.167 GHz on a ES-DVT4 Spectrometer. Thermal analyses of the complexes were measured under N\(_2\) atmosphere with heating rate of 20 °C/minute on a Schimadzu Thermal using alumina as reference. The DPPH radical was evaluated using a UV-visible spectrophotometer (UV 1601; Shimadzu, Japan) at 517 nm. The SEM images of the macrocyclic compounds at 3000 magnification (voltage 15-20 kV) were taken using Scanning Electron Microscope JEOL-JAPAN, equipped with an energy dispersive X-Ray spectrooscope EDAX with 20 kV accelerating voltage.

5.2.3 Molar conductivity measurements
The complexes were soluble in DMF and DMSO. All the complexes (10\(^{-3}\) mol L\(^{-1}\)) exhibited low range of molar conductance indicating their non electrolytic nature (10-27 cm\(^2\) Ω\(^{-1}\) mol\(^{-1}\)) in DMSO.

5.2.4 Tetrachloro[1, 1' biphenyl bis(1,6,9,14-tetrahydro-3,4;11,12-diphenyl-1,6,9,14-tetraazacyclohexadecane)] di metal(II).nH\(_2\)O, [M\(_2\)LCl\(_4\)].nH\(_2\)O where M = Co(II), Ni(II) and Zn(II)

The complexes were synthesized by metal ion controlled reaction between 3,3'-
diaminobenzidine with 1,2-bis(bromomethyl)benzene, ethane-1,2-diamine in presence of KOH. The hot solutions of 3,3'-diaminobenzidine (0.214 g; 1.0 mmol, in 20 mL methanol) and ethane-1,2-diamine (0.122 g; 2 mmol, in 20 mL methanol) were added simultaneously to the solution of the respective metal (II) chloride (2 mmol, in 10 mL methanol) which were magnetically stirred at 65 °C. The resulting dark color solution was refluxed on water bath for 1 hour and subsequently hot solution of 1,2-bis(bromomethyl)benzene (1.05 g; 4 mmol, in 20 mL methanol) with KOH was added. The refluxing was done for 15 h leading to the isolation of solid product. The product thus formed was filtered, methanol washed and finally dried in vacuum over anhydrous CaCl₂ (Scheme 5.1).

5.2.5 Tetrachloro [1, 1' biphenyl bis(1,6,9,14-tetrahydro-3,4;11,12-diphenyl-1,6,9,14-tetraazacyclohexadecane)] di copper(II), [Cu₂LCl₄]

The complex (3) was synthesized by adopting the same procedure as described above.

[C₄₈H₅₄N₈Cl₄Cu₂].2H₂O (1): Yield: 58%. Color: Dark brown. Anal. Calc. for C₄₈H₅₄N₈Cl₄Cu₂.2H₂O (MW: 1038.70): C, 55.50; H, 5.62; N, 10.78; Found: C, 55.62; H, 5.70; N, 10.83%. IR (KBr, cm⁻¹): 3270 v(N-H), 3029 v(C-H), 1160 v(C-N), 530 v(M-N) and 3455 v(H₂O). El-Mass: (m/z, [M + Na⁺]) 1062.89; Λm/Ω -1 cm² mol⁻¹ (1 × 10⁻³ M, DMSO): 27, m.p.: 410 °C.

[C₄₈H₅₄N₈Cl₄Ni₂].2H₂O (2): Yield: 53%. Color: Greenish brown. Anal. Calc. for C₄₈H₅₄N₈Cl₄Ni₂.2H₂O (MW: 1038.22): C, 55.52; H, 5.63; N, 10.79; Found: C, 55.60; H, 5.73; N, 10.81%. IR (KBr, cm⁻¹): 3293 v(N-H), 3032 v(C-H), 1169 v(C-N), 540 v(M-N) and 3488 v(H₂O). El-Mass: (m/z, [M + H⁺]) 1039.32; Λm/Ω -1 cm² mol⁻¹ (1 × 10⁻³ M, DMSO): 10. m.p.: 390 °C.

[C₄₈H₅₄N₈Cl₄Cu₂] (3): Yield: 47%. Color: Dark green. Anal. Calc. for C₄₈H₅₄N₈Cl₄Cu₂ (MW: 1011.89): C, 56.97; H, 5.37; N, 11.07; Found: C, 56.79; H, 5.28; N, 11.17%. IR (KBr, cm⁻¹): 3381 v(N-H), 3078 v(C-H), 1182 v(C-N) and 516 v(M-N). El-Mass: (m/z, [M + H⁺]) 1012.62; Λm/Ω -1 cm² mol⁻¹ (1 × 10⁻³ M, DMSO): 21. m.p.: 342 °C.

[C₄₈H₅₄N₈Cl₄Zn₂].H₂O (4): Yield: 68%. Color: Red brown. Anal. Calc. for C₄₈H₅₄N₈Cl₄Zn₂.2H₂O (MW: 1033.57): C, 55.77; H, 5.65; N, 10.84; Found: C, 55.86; H, 5.70; N, 10.92%. IR (KBr, cm⁻¹): 3267 v(N-H), 3024 v(C-H), 1127 v(C-N), 470 v(M-N) and 3510 v(H₂O). El-Mass: (m/z, [M + Na⁺]) 1057.41; Λm/Ω -1 cm² mol⁻¹ (1 × 10⁻³ M, DMSO): 17. m.p: 376 °C.
Scheme 5.1 Schematic illustration of macrocyclic complexes of Co(II), 1; Ni(II), 2; Cu(II), 3; and Zn(II), 4.

5.3 CHARACTERIZATION

5.3.1 Antioxidant activity

The property of complexes to bleach the stable radical DPPH is exclusive factor for the quantitative estimation of the free radical scavenging activity. Briefly, varying concentration (50-450 µg/mL) of compounds were taken and added to methanolic solution of DPPH (3 mL) with final concentration 0.1 mM. As positive control, ascorbic acid was used. The reaction mixture was stirred and kept for about 30
minutes under dark conditions. Thereafter, calculation of DPPH radical reduction was made by UV-visible spectrophotometer (517 nm). The methanolic solution of DPPH without the sample served as a control.

The following equation was used to calculate percentage inhibition:

\[
\% \text{Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

where

\[A_{\text{control}} = \text{the absorbance of DPPH in methanol.}\]

\[A_{\text{sample}} = \text{the absorbance of DPPH in presence of an antioxidant.}\]

5.3.2 Bacterial strains used for the evaluation of the complexes antibacterial potential

*S. aureus* (MTCC no. 3160), *L. monocytogenes* (MTCC no. 3906) Gram positive, *E. coli* (MTCC no. 1652), *P. aeruginosa* Gram negative (MTCC no. 4676) were used to study the antibacterial potentialities of macrocyclic complexes. *S. aureus* and *L. monocytogenes* were cultured in Brain Heart Infusion broth while *E. coli* and *P. aeruginosa* strains were cultured in Luria Bertani broth medium at 37 °C for 12-18 h.

5.3.2.1 Antibacterial assessments

The complexes (1-4) were investigated for the antibacterial potential using agar well diffusion method as reported previously.\(^{22}\) The dilution of the overnight cultures was performed with the inoculum made with sterile normal saline to a 0.5 McFarland standard. The preparation of agar petri plates were done by spreading mature broth culture of specific bacterial strains (1 × 10^8 CFU/mL) with a sterile glass rod. With the help of gel puncture, the well was punctured in each petri-plate. Now, the stock solution of each compound formed at a concentration of 1 mg/mL, suspended in sterile PBS for antibacterial activity. The experiment was done under sterile conditions using level-2 bio-safety hoods with overnight incubation at 37 °C. The susceptibility was determined depending on the inhibition zone developed for various pathogenic strains (*S. aureus*, *L. monocytogenes*, *P. aeruginosa* and *E. coli*). The antibacterial study was performed in duplicate and the mean value was calculated and compared with the reference drug vancomycin. In parallel, antibacterial nature of synthesized compounds was evaluated by means of micro dilution using 96 micro well plates, according to Clinical and Laboratory Standards Institute procedures against the aforementioned gram positive and gram negative bacterial strains pursuance to the previously developed protocols.\(^{22}\)
5.3.3 *E. coli* and *S. aureus* viability assay using propidium iodide (PI) staining

Fluorescence microscopic studies were made on a Zeiss microscope for detection of cell killing by the complexes (1-4). *S. aureus* and *E. coli* cultures were labeled with propidium iodide (PI). PI was used to estimate the dead cells as PI specifically stains only dead bacteria. The compound at their MICs was added to the overnight grown bacterial cultures and incubated at 37 °C for 120 minute. From both of these bacterial strain cultures, after centrifuging at 2500xg for 5 min, 1 mL volumes were harvested. The pellet was resuspended in 1 mL PBS buffer. Staining with PI was performed by adding 10 µL of PI to a desired concentration of 1 µg/mL. This solution was incubated for 3 h at 37 °C. Likewise a control of both bacterial strains (not treated with compounds) was grown under same conditions. A drop of the above prepared samples was then placed on a glass slide and mounted with the cover slip then cells were examined under a fluorescence microscope.23

5.3.4 Transmission electron microscopy of bacterial cells

*E. coli* and *S. aureus* cultured in the modified-LB medium were exposed to all the synthesized complexes for the time period of 60 minutes with constant agitation at 37 °C at 200 rpm. The cell suspension was washed 5 times in modified-LB medium and 3 times in PBS to remove unbound or loosely associated particles of synthesized compound. The bacterial suspension (approximately 10^8 CFU) that interacted with synthesized compounds at stipulated time interval, were prepared and imaged using TEM. Briefly, the bacteria, (~10^9 CFU) were fixed with 1% glutaraldehyde in PBS and subsequently exposed to 1% osmium tetraoxide in water, for 24 hours each. The sample was transferred onto a 0.22 µm pore size filter (Millipore) and substituted with acetone, and subsequently with liquid CO_2 in a critical point drying apparatus (SPI, USA). Filter paper sections were metalized with gold, by sputter coating, and imaged with a JEOL JSM-6390 LV.24

5.3.5 XTT Biofilm assay

To evaluate the antibiofilm activity of synthesized compounds, the XTT based biofilm assay was executed.25 Briefly, the *S. aureus* was allowed to form their mature biofilms on a 96 well plate for 48 h and the plates were washed with sterile PBS to remove non adherent cells. The mature biofilms were treated with varying concentration of different formulations and placed at 37 °C for 48 h. After stipulated incubation period, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-((phenylamino) carbonyl)-2H-tetrazolium
hydroxide (XTT) solution in PBS, was added at a final concentration of 250 mg/mL. The obtained solution was filtered and sterilized using a 0.22-mm pore-size filter and stored at -80 °C until required. Menadione solution (0.4 mM) was made followed by immediate filtration, just before the commencement of each assay. Adherent cells were washed with PBS (200 µL), and XTT was added, with addition of 2 µL of menadione to each well. After incubating in the dark for 4 h at 37 °C, the solution was transferred to a new plate and the colorimetric change in the solution was assessed using a microtiter plate reader (BIORAD Microplate reader at 490 nm). Experiments were performed in duplicate.

5.3.6 Sample preparation of stock solutions
For the preparation of 20 mM phosphate buffer (pH = 7.4), doubly distilled water was used. By dissolving the desired amount of HSA in 20 mM phosphate buffer (pH = 7.4), a stock solution of HSA was made. The HSA stock solution was stored at 4 °C in the dark and used within 2 h. Its concentration was evaluated on a Perkin-Elmer λ-25 spectrometer using the molar absorption coefficient using $E_{1\%}^{1\text{cm}}$ of 5.3 at 280 nm. Drug solution of 2 mM concentration was made in 20 mM phosphate buffer (pH = 7.4) and diluted to desired concentrations in the same buffer.

5.3.7 HSA binding experiments
5.3.7.1 Fluorescence quenching measurement of HSA
In fluorescence quenching experiments, HSA (5 µM) was titrated using varying concentration of compounds (0-45 µM) in 20 mM of phosphate buffer at pH 7.4. The fluorescence emission spectra were recorded on F-2700 Fluorescence spectrophotometer (HITACHI) in range of 300-500 nm with a quartz cuvette of 1.0 cm path length after setting slit width of both excitation and emission at 10 nm. To minimize the effect of tyrosine residues in the emission signal, all the samples were excited at 295 nm. For correction of background of fluorescence, appropriate blanks corresponding to the buffer were subtracted. In each experiment, after addition of the complexes, the mixture was allowed to incubate for 2 min. The intensity at 340 nm (tryptophan) was used to determine the Stern-Volmer quenching constant ($K_{sv}$).

5.3.7.2 Calculations of energy transfer
The efficiency of energy transfer (E) can be used to measure the distance between the
fluorophores in the protein (donor) and metal complex (acceptor). According to Föster’s non-radiative energy transfer theory, the energy transfer depends on (i) the donor (protein) should have fluorescence light (ii) the fluorescence emission spectrum of the donor and the UV absorption spectrum of the acceptor should have overlap with each other and (iii) the average distance between the donor and the acceptor pair should be below 8 nm. The efficiency of energy transfer (E) could be evaluated by following equation:

\[ E_{FRET} = \frac{R_0^6}{(R_0^6 + r^6) - 1 - \frac{F}{F_0}} \]  ---- (5.2)

where F and F₀ are the fluorescence intensities of HSA in the presence and absence of metal complex, r is the distance between acceptor (bound metal complex) and donor (Trp-214 residue of HSA) and R₀ is the critical distance when the transfer efficiency is 50%. The value of R₀ was calculated using the following equation:

\[ R_0^6 = 8.8 \times 10^{-25}K^2n^{-4}\Phi J \]  ---- (5.3)

In the above equation, \( K^2 \) = spatial orientation factor describing the relative orientation in space of the transition dipoles of the donor and acceptor, n = refractive index of the medium, F = fluorescence quantum yield of the donor and J = overlap integral of the fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor which can be calculated by equation:

\[ J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda/\sum F(\lambda)\Delta\lambda \]  ---- (5.4)

where, the parameters F(λ) denotes the corrected fluorescence intensity of the donor at wavelength λ and ε(λ) is the UV molar absorption coefficient of the acceptor at the wavelength λ.

Under the experimental condition for HSA, the value of \( K^2 = 2/3 \), \( N = 1.336 \) and \( F = 0.188 \).

5.3.7.3 Investigation of HSA conformational changes using circular dichroism (CD) spectra

The circular dichroism measurements were made on a JASCO spectrometer (J-815) with a thermostatically controlled cell holder attached to a peltier device having water circulator. All measurements were carried out at 298 K keeping the HSA concentration constant (5 µM) while varying complexes (1-4) concentrations. The molar ratio of protein-complex was taken as 1:0, 1:2, 1:4 and 1:6. The spectra were scanned in range of 200-250 nm using cuvette of a 0.1 cm path length. The bandwidth was kept at 1 nm with a scanning speed of 100 nm/min and response time of
1 sec. Each spectrum was baseline corrected and the final plot was taken as an average of the two accumulated scans.

5.3.8 Molecular docking

In order to investigate more details of binding, we performed molecular docking using HEX 8.0.0 software\textsuperscript{28} which is defined as an interactive molecular graphics program for calculating and displaying feasible docking modes of pairs of protein, DNA molecule and enzymes. The CHEMSKETCH (http://www.acdlabs.com) was use to draw the structure of compounds and converted to pdb format from mol using OPENBABEL (http://www.vcclab.org/lab/babel/). The three dimensional crystal structure of HSA was downloaded from RCSB Protein Data Bank in pdb format [PDB: 1AO6] utilized as a template. All the water molecules surrounding of HSA were removed to avoid hindrance in docking process.\textsuperscript{29} All other docking parameters were kept as default. PyMol (http://pymol.sourceforge.net/) and CHIMERA (www.cgl.ucsf.edu/chimera) were used for visualization purpose. The docked conformation with the lowest energy was selected for analysis.

5.4 RESULTS AND DISCUSSION

The analytical, spectral, thermal stability and SEM studies were performed for the stoichiometric, geometric, thermal and morphological nature of the synthesized binuclear macrocyclic complexes. The antibacterial analysis was made using different \textit{in-vitro} studies. Furthermore, antibiofilm and antioxidant activity have been performed. In the present work, the drugs interaction with HSA has been focused which can explicate the numerous applicabilities of drug-protein complex that may provide valuable informations related to absorption, transportation, distribution and metabolism which establish the therapeutic efficacy of drugs. The HSA binding capability of these complexes was studied using different biophysical and molecular docking studies.
5.4.1 FT-IR analysis

The important FT-IR spectral features for all the complexes (1-4) were shown in Figure 5.1.

![FT-IR spectra of Co(II), Ni(II), Cu(II) and Zn(II) complexes.](image)

Figure 5.1 FT-IR spectra (1-4) of Co(II), Ni(II), Cu(II) and Zn(II) complexes.

The presence of single sharp band in the region 3270-3320 cm\(^{-1}\) corresponds to the coordinated \(\nu\)(N-H) vibration of a secondary amine moiety.\(^{34}\) The formation of bis(macroyclic) bimetallic complexes have been confirmed by the absence primary amine or bromo group bands expected from either ethane-1,2-diamine/3,3'-diaminobenzidine or 1,2-bis(bromomethyl)benzene, respectively. The appearance of strong-intensity band in region 1560-1640 cm\(^{-1}\) is assigned to \(\delta\)(N-H) vibrations for secondary amine. All the complexes displayed a strong band in the region 1127-1182 cm\(^{-1}\) assigned to the \(\nu\)(C-N) group. Whereas, bands in region 2850-2960 cm\(^{-1}\) correspond to \(\nu\)(C-H) vibrations. The bands appeared in region 735-1480 cm\(^{-1}\) were attributed to phenyl group vibrations. The \(\nu\)(M-N) stretching vibration was marked by the presence of peaks in the region 470-540 cm\(^{-1}\).\(^{30}\) While medium-intensity bands observed in the range of 270-300 cm\(^{-1}\) in case of all the metal complexes, may be assigned to \(\nu\)(M-Cl) vibrations. The presence of water molecules in case of the complexes (1, 3 and 4) was authenticated with the appearance of \(\nu\)(OH) band in the region 3455-3510 cm\(^{-1}\).\(^{31}\)
5.4.2 $^1$H NMR and $^{13}$C NMR spectrum

In the $^1$H NMR spectrum of zinc(II) complex, no signal was observed for primary amino protons of 3,3′-diaminobenzidine moiety at 4.57 ppm which strongly supports the complex formation. The characteristic signals obtained in region 3.92-4.09 and 4.24-4.28 ppm (m, 4H; -C-NH- and t, 4H; -C-NH-) may correspond to the -NH group of the macrocyclic complex [Figure 5.2 (a)]. The spectrum displayed the multiplets in region 7.09-7.49 ppm (m, 22 ArH) ascribed to aromatic protons of 3,3′diaminobenzidine and 1,2-bis(bromomethyl)benzene moiety. However, two different signals in region 3.27-3.29 and 4.71-4.72 ppm (d, 8H; -C-CH$_2$N- and d, 8H; -N-CH$_2$-) assigned to middle methylene protons of the scaffolds adjacent to the aromatic ring. Further, another multiplet observed in region 2.46-2.49 ppm (m, 8H; -N-CH$_2$-) may be assignable to methylene protons of the ethane-1,2-diamine moiety. An additional signal observed at 3.62 ppm may ascribe to the water molecule.

Figure 5.2(a) $^1$H NMR spectrum of macrocyclic complex of Zn(II), 4.
The structure of Zn(II) complex was also confirmed by $^{13}$C NMR spectrum. The signals appearing in region 118.55-152.07 ppm may reasonably be assigned to aromatic carbons.\(^{35}\) whereas the signals observed at 52.16 and 50.47 ppm were attributed to the methylene carbons adjacent to the aromatic ring\(^ {36}\) [Figure 5.2 (b)]. Moreover, the resonance signal observed at 44.28 ppm was assigned for methylene carbons of ethane-1,2-diamine moiety.\(^ {30}\)

![Figure 5.2(b) $^{13}$C NMR spectrum of macrocyclic complex of Zn(II), 4.](image)

**5.4.3 Mass spectral studies**

The synthesized macrocyclic complexes (1-4) were analyzed by mass spectroscopy and the molecular ion peaks appeared at m/z, [M + Na]$^+$/[M + H]$^+$ values with different intensities. The mass spectra contain molecular ion peak at m/z 1062.89 ([M + Na]$^+$; complex 1), 1039.32 ([M + H]$^+$; complex 2), 1012.62 ([M + H]$^+$; complex 3) and 1057.41 ([M + Na]$^+$; complex 4), respectively which confirmed their proposed molecular formulae. Figure 5.3 display representative spectra of complexes 1, 2, 3 and 4.
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(a)

(b)
Figure 5.3 Mass spectra of Co(II), Ni(II), Cu(II) and Zn(II) complexes (a-d).
5.4.4 EPR spectroscopy

The nature of the bonding between a metal ion and its ligands along with the elucidation of their complexes geometry can be determined using EPR spectroscopy. The complexes with one or more unpaired electrons are particularly studied using this technique. The absence of hyperfine signal in the EPR spectrum of Cu(II) complex (Figure 5.4) may reflect the strong dipolar and exchange interaction between Cu(II) ions in the unit cell. The spectral data revealed that the observed axial symmetric g-tensor value of Cu(II) complex exhibit the order as: \( g_{II} \) (2.09) > \( g_{\perp} \) (2.07) > \( g_{e} \) (2.0023). This observation inferred that \(^2\!B_{1g}\) has ground state with an unpaired electron in the dx\(^2\)-y\(^2\) orbital.

![EPR spectrum](image)

**Figure 5.4 EPR spectrum of Cu(II) complex.**

The distorted octahedral geometry for Cu(II) ion was observed in view of the fact that \( g_{II} > g_{\perp} \) (2.09 > 2.07).\(^ {38}\) The value of \( g_{av} \) (2.07) was calculated using the following expression:

\[
g_{av}^2 = g_{II}^2 + 2g_{\perp}^2/3
\]

----- (5.5)

Also, to measure the exchange interaction between the metal centers in polycrystalline solid, the geometric parameter, \( G \) was calculated using the equation:

\[
G = \frac{g_{II} - 2}{g_{\perp} - 2}
\]

----- (5.6)
According to Hathway, the value of $G < 4.0$, suggest the presence of sufficient exchange interaction in the complex. However, $G > 4.0$ signifies negligible exchange interaction.\textsuperscript{39} The observed $G$ value of the complex (1.28) suggesting the exchange interaction between Cu(II) centers in the solid state.\textsuperscript{40} Notably, $g_{II}$ is normally 2.0023 or higher for covalent surroundings, whereas $g_{II} < 2.0023$ for a ionic environment.\textsuperscript{41} The obtained $g_{II}$ is greater than 2.0023, thus confirming the significant covalent behavior of the Cu(II)-ligand bond.\textsuperscript{42}

5.4.5 \textit{Electronic absorption spectral studies and magnetic moments}

The electronic spectral bands with magnetic moment of binuclear metal complexes (1-3) are summarized in \textit{Figure 5.5, Table 5.1}.

\textit{Figure 5.5 The solution state UV-vis spectra of complexes (1-3).}
Table 5.1 Magnetic moment and electronic transitions of the complexes (1-3) with their band assignments.

<table>
<thead>
<tr>
<th>Complexes</th>
<th>Magnetic moment (B.M)</th>
<th>Band positions (cm$^{-1}$)</th>
<th>Band assignments</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>4.58</td>
<td>13623 18214 28653 38610</td>
<td>$^4 A_{2g}(F) \leftarrow ^4 T_{1g}(F)$  $^4 T_{1g}(P) \leftarrow ^4 T_{1g}(F)$  $n \rightarrow \pi^<em>$  $\pi \rightarrow \pi^</em>$</td>
<td>849 945 1320 1704</td>
</tr>
<tr>
<td>(2)</td>
<td>3.12</td>
<td>13661 20790 27777 35714</td>
<td>$^3 T_{1g}(F) \leftarrow ^3 A_{2g}(F)$  $^3 T_{1g}(P) \leftarrow ^3 A_{2g}(F)$  $n \rightarrow \pi^<em>$  $\pi \rightarrow \pi^</em>$</td>
<td>673 770 1114 1533</td>
</tr>
<tr>
<td>(3)</td>
<td>1.71</td>
<td>16977 21691 26525 37593</td>
<td>$^2 B_{2g} \leftarrow ^2 B_{1g}$  $^2 E_g \leftarrow ^2 B_{1g}$  $n \rightarrow \pi^<em>$  $\pi \rightarrow \pi^</em>$</td>
<td>525 592 806 1021</td>
</tr>
</tbody>
</table>
The electronic spectrum in case of Co(II) complex, 1 exhibited two broad bands at 13,623 and 18,214 cm\(^{-1}\) consistent with \(^4\)A\(_{2g}\)(F)\(\leftrightarrow\)^4T\(_{1g}\)(F), \(^4\)T\(_{1g}\)(P)\(\leftrightarrow\)^4T\(_{1g}\)(F) transitions, respectively correlated with the octahedral geometry around the Co(II) ion.\(^{43}\) However the spectrum of Ni(II) complex, 2 revealed medium intensity absorption bands at 13,661 and 20,790 cm\(^{-1}\) ascribed to \(^3\)T\(_{1g}\)(F)\(\leftrightarrow\)^3A\(_{2g}\)(F) and \(^3\)T\(_{1g}\)(P)\(\leftrightarrow\)^3A\(_{2g}\)(F) transitions, respectively, signifying its octahedral geometry.\(^{44}\) While the electronic spectrum of the Cu(II) complex, 3 showed two absorption bands at 16,977 and 21,691 cm\(^{-1}\) corresponding to \(^2\)B\(_{2g}\)\(\leftrightarrow\)^2B\(_{1g}\) and \(^2\)E\(_g\)\(\leftrightarrow\)^2B\(_{1g}\) transitions, respectively consistent with a distorted octahedral geometry.\(^{45}\) The above mentioned complexes (1-3) showed high intensity bands in the range of 35,714-38,610 and 26,525-28,653 cm\(^{-1}\) which may be associated with the intra ligand charge transfer transition of π→π* and n→π* group, respectively.

The observed magnetic moment for Co(II) complex, 4.58 B.M, corresponds to high spin octahedral complexes. The magnetic moment of the Ni(II) complex was found to be 3.12 B.M which showed the presence of octahedral environment around the metal atom.\(^{46}\) The magnetic moment of Cu(II) complex was found to be 1.71 B.M which lies within the permissible range recorded for one unpaired electron consistent with an octahedral environment.\(^{47}\)

**5.4.6 Thermal analysis**

To gain the information about the thermal behaviour of the synthesized metal complexes (1-4), stepwise thermal degradation in different temperature range was recorded (Figure 5.6).
Figure 5.6 TGA/DTA curves of complexes 1-4 (a-d).
In case of complexes 1 and 2, the estimated mass loss was observed to be 3.41% (calcd. 3.46%) and 3.42% (calcd. 3.47%), respectively within the temperature range of 30-140 °C and could be attributed to the loss of two crystalline water molecule which was further corroborated with the endothermic DTA curve peak in range of 50-130 °C. While no weight loss was observed upto 150 °C during the degradation of complex 3 suggesting the absence of any lattice/crystalline water molecule which was further confirmed by the absence of endothermic peak in this temperature range. However, the thermal behaviour complex 4 exhibited weight loss of 1.89% (calcd. 1.74%) in range 30-130 °C, signifying the loss of only one crystalline water molecule. This was further corroborated with the presence of endothermic DTA peak in the range of 30-100 °C. In case of complexes 1, 2 and 4, the water molecules are not coordinated to the metal ion as inferred from the temperature at which they are lost. Due to the elevation in temperature, the second step of degradation for complexes (1-4) was displayed in range 150-320 °C owing to the elimination of four chlorides ions with approximate weight loss of 13.92% (calcd. 13.65%), 13.15% (calcd. 13.65%), 14.28% (calcd. 14.01%) and 13.92% (calcd. 13.71%), respectively. The endothermic DTA peaks in range 220-290 °C further confirm the above mentioned decomposition of the complexes (1-4). The third step weight loss for complexes (1-4), displayed in range 330-580 °C was attributed to the thermal decomposition of organic moiety along with the bridging molecule i.e. diaminobenzidine with loss of 72.15% (calcd. 71.53%), 71.05% (calcd. 71.56 %), 74.28% (calcd. 73.42%) and 72.15% (calcd. 71.88%), respectively. The presence of exothermic DTA peaks in range 450-560 °C further correlated well with the above stated decomposition in case of the complexes (1-4). Moreover, there was no weight loss beyond 640 °C implicating metal oxides as final residues in case of all the complexes.

5.4.7 SEM analysis

Figure 5.7 displayed the comparative morphological analysis of macrocyclic complexes (1-4). In case of Co(II) complex (1), unevenly distributed heterogeneous range of structural features with smooth surface was observed. While SEM image of Ni(II) complex (2), revealed non-uniform agglomerated pattern. In addition, Cu(II) complex (3), showed somewhat floral appearance which were distributed unevenly. Whereas, the micrograph of Zn(II) complex (4), depicts variable dimensions of broken plates which was scattered irregularly.
5.4.8 Results of biological study

5.4.8.1 DPPH free-radical scavenging assay

There is wide variety of sources which results in the production of highly reactive free radicals and oxygen species. These free radicals may initiate degenerative disease and can oxidize nucleic acids, proteins, lipids or DNA.\textsuperscript{50} To explore the free radical scavenging nature of the complexes (1-4), the experiments have been carried out to design potential antioxidants. The inhibition percentage of all the synthesized complexes (1-4) obtained from DPPH method at different concentration was less potent than ascorbic acid as the reference. The results vary in a concentration dependent manner (Figure 5.8).
Figure 5.8 Antioxidant activities of compounds (1-4) with ascorbic acid (standard) against DPPH radical at different concentrations.

All the analyses were made in duplicate and the results were averaged suggesting that the complexes (1-4) are less potent compared to ascorbic acid (reference). The highest inhibition percentage monitored in complex 3 in comparison to the other complexes (1, 2 and 4) was 70%, which is lower than that of reference at 450 µg/mL (80%). The activity difference of complexes may be accredited due to the coordination nature and redox properties. In general, there are several factors such as axial ligation, charge type, coordination number and chelate ring size, unsaturation degree and chelate ring substitution pattern are responsible for the complex redox properties. Thus, these metal complexes could be beneficial in improving their availability for therapeutic purposes.

5.4.8.2 Antimicrobial activity: Agar well-diffusion method and MIC analysis

There are different approaches for the in-vitro antibacterial activity assessment. In the present work, the agar diffusion assay was used to investigate the bacterial inhibition. The conventional antibiotic Vancomycin was taken as control. It is evident from these results that all the metal complexes possess effective antibacterial nature when tested against gram -ve as well as gram +ve bacterial strains (Figure 5.9). The average value of zone of inhibition for the complexes (1-4) was recorded as given in Table 5.2.
Figure 5.9 Zone of inhibition measured by disc diffusion assay after the treatment with complexes (1-4) and (A) and (B) represent S. aureus and L. monocytogenes while (C) and (D) represent P. aeruginosa and E. coli bacterial strains, respectively (A1). The diameter of the zone of inhibition (in mm) of complexes (1-4) against gram positive and gram negative bacterial strains (A2).
Table 5.2 The inhibitory effects (in mm) of complexes (1-4) against bacterial strains using disk diffusion.

<table>
<thead>
<tr>
<th>Complexes</th>
<th>Zone of inhibition (in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Gram+ve)</td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>(1)</td>
<td>12.50</td>
</tr>
<tr>
<td>(2)</td>
<td>12.50</td>
</tr>
<tr>
<td>(3)</td>
<td>15.50</td>
</tr>
<tr>
<td>(4)</td>
<td>15.50</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>16.50</td>
</tr>
</tbody>
</table>

However, the complex 3 showed significant activity with the zone of inhibition values as 15.5, 15, 17.5 and 19 mm against *S. aureus*, *L. monocytogenes*, *P. aeruginosa* and *E. coli*, respectively, in comparison to the other synthesized complexes. These results were further validated by evaluating MIC’s for synthesized complexes as MIC is generally regarded as a significant parameter in diagnostic laboratories to ascertain sensitivity of a microorganism against a specific antimicrobial agent. The antibacterial effect of complexes (1-4) was determined against gram +ve and gram -ve bacterial strains using the micro-dilution method. As shown in Table 5.3, the complex 3 showed better MIC values for both *S. aureus* and *E. coli* (32 µg/mL), whereas 64 and 16 µg/mL for *L. monocytogenes* and *P. aeruginosa* strains, respectively.

The above stated results conferred that the compounds (1-4) exhibit satisfactory potency against tested pathogens compared to Vancomycin and may be used as an antibacterial drug with modifications. The complexes antibacterial behavior with respect to structural changes and reactivity, induced by *in-situ* coordination of ligand moiety with the metal ion may be understood in terms of chelation theory.\textsuperscript{52}
**Table 5.3** The minimum inhibitory concentration (in μg/mL) of complexes (1-4) against bacterial strains using micro broth dilution method.

<table>
<thead>
<tr>
<th>Complexes</th>
<th>MIC (μg/mL)</th>
<th>(Gram+ve)</th>
<th>(Gram-ve)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S. aureus</td>
<td>L. monocytogenes</td>
</tr>
<tr>
<td>(1)</td>
<td></td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>(2)</td>
<td></td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>(4)</td>
<td></td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td>12.50</td>
<td>3.25</td>
</tr>
</tbody>
</table>

5.4.8.3 **Bacterial viable/dead cells assessment**

To further prove the antibacterial nature of complexes against *S. aureus* and *E. coli* strains, the viable/dead cells assay was performed with fluorescence microscopy using PI. Bacterial cells were grown and stained with PI after their exposure to compounds for three hours. PI penetrates only those cells which have severe membrane lesions; since, it is known that PI, as a nucleotide binding strain, is incapable to stain the membrane of the cytoplasm of the cells. It intercalates into the double-stranded nucleic acids. The PI uptake for the tested compounds (1-4) [Figure 5.10(A1-A2)] showed that the control cells were live and displayed no red fluorescence whereas treated cells (for 3 h) showed good red fluorescence confirming the concept of invading cells, thus warranting that the synthesized complexes damage the bacterial cell wall since the PI is binding to the double stranded DNA.
Unstained live cells

(1) Stained live cells

(2)

(3)

(4)
Figure 5.10 Fluorescence microscopic observations of E. coli (A1) and S. aureus (A2) with PI staining for complexes (1-4), respectively.
5.4.8.4 TEM analysis for the interaction of compound (3) with bacteria

The interaction between bacterial cells and the synthesized compound was determined using TEM analysis. The damaged cell wall and cell membrane were depicted from the ultrathin sections of treated cells [Figure 5.11]. The metal complexes undergoes interaction with cell membrane and adhere onto the bacterial surface leading to disorganized cell permeability and eventually resulting in cell death.  

Figure 5.11 Electron microscopic observations of E. coli (a) and S. aureus (b) for complex 3.

5.4.8.5 Antibiofilm potential of compounds (1-4)

The S. aureus strains are known to produce exopolysaccharide that exhibited vital role in the bacterial adhesion to host cells and the expansion of a complex biofilm structure which is then challenging to contest with host defenses and antibiotics. The anti-biofilm activity could be because of restriction of exopolysaccharide formation in view of the fact that metal complexes damage exopolysaccharide synthesis thus limiting the formation of biofilm. The XTT assay was employed for their antibiofilm potential of as-synthesized compounds which revealed a varying level of depletion of biofilm by S. aureus in the vicinity of complexes (1-4) in a concentration-dependent manner (Figure 5.12). The comparative study suggested that complex 3 was able to restrict the biofilm formation more effectively.
Figure 5.12 Effect of compounds (1-4) on inhibition of biofilm development in S.aureus. Biofilm growth inhibition was determined by comparing relative metabolic activity (RMA) obtained through XTT assay.

5.4.8.6 Tryptophanyl fluorescence quenching

The HSA primary sequence reveals that the protein is a single polypeptide consisting of 585 residues with disulfide bridges in 17 pairs and one free cysteine and contains three structurally homologous domains (I, II, and III), each containing two subdomains (A and B), probably derived through gene multiplication. In HSA, the relative proportions of α-helix, β-structure, and random coil were 65%, 6%, and 29%, respectively. To explore the interactions between the drug molecule and albumins, fluorescence spectral study is an effective approach. The intrinsic fluorogenic properties of HSA significantly contributed by three fluorophores namely tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) moieties where tryptophan has strongest fluorescence emissions. The phenylalanine has low quantum yield and the fluorescence of tyrosine is nearly quenched, if it is ionized or near an amino group or tryptophan residues. In general, HSA has one tryptophan residue (Trp-214) that take part in additional hydrophobic interactions at the IIA-III interface. Trp-214 in HSA is located in a similar hydrophobic microenvironment (sub-domain IIA). Interaction of a drug and protein affects the absorption, distribution and elimination of drug in the circulatory system. In addition, this binding may restrict fast elimination of drugs from bloodstream and enhancing plasma drug solubility, reducing its toxicity; prolong
its *in-vivo* half-life and protecting from oxidation. The synthesis and design of new drugs necessitate the application of HSA-binding study that encouraged us to inspect HSA-binding behavior of macrocyclic metal complexes. In the absence and presence of each complex, the strong quenching of HSA fluorescence spectra (quencher) were scrutinized at the wavelength of 290 nm where a strong fluorescence emission peak was observed at 340 nm due to the emission from the single tryptophan residue (Trp-214). Upon the succeeding addition of increasing concentration of each complex (0-45 µM) to the fixed amount of HSA (5 µM), resulted in the reduction in fluorescence intensity of complexes 1-4 as 50.56%, 58.54%, 62.92% and 47.96%, respectively [Figure 5.13(A1)].

![Fluorescence quenching spectra of HSA with complexes (1-4) induced by different molar ratios of complexes to HSA in 20 mM sodium phosphate buffer (pH 7.4) at 298 K upon excitation at 295 nm. The concentration of HSA was 5 µM and the concentration of compounds was 5-45 µM (a-d).](image)

*Figure 5.13 (A1): The fluorescence quenching spectra of HSA with complexes (1-4) induced by different molar ratios of complexes to HSA in 20 mM sodium phosphate buffer (pH 7.4) at 298 K upon excitation at 295 nm. The concentration of HSA was 5 µM and the concentration of compounds was 5-45 µM (a-d).*
It is evident from these results that each complex quenched well in an order of \(3 > 2 > 1 > 4\) leading to conformational alteration in HSA. The quenching results inferred that the protein binding ability of metal complexes incited conformational alterations in HSA as the intramolecular forces responsible to sustain the structure could be changed (affecting the tryptophan residues of HSA) along with decreased hydrophobicity. This demonstrates more exposure of Trp residues to the solvent. However, HSA fluorescence quenching mechanism in presence of each complex was analyzed via Stern-Volmer equation.\(^{62}\)

\[
\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + k_q[Q]
\]

\[\text{(5.7)}\]

where \(F_0\) and \(F\) refer to the HSA fluorescence intensity in absence and presence of the complexes. \(K_{sv}\) is the Stern-Volmer quenching constant, \(k_q\) is bimolecular rate constant of the quenching reaction, \(\tau\) is related to average fluorescence life time of tryptophan which is typically equal to \(10^{-8}\) s for biomacromolecules.\(^{63}\) The calculated \(K_{sv}\) values of the complexes (1-4) using slope of Stern-Volmer equation was obtained and presented in Figure 5.13(A2) and Table 5.4.

![Stern-Volmer plots](image)

**Figure 5.13 (A2) Stern-Volmer plot (a-d) for HSA in presence of compounds (1-4).**
The Stern-Volmer plot only authenticates quenching process but not its type i.e., static or dynamic quenching.\textsuperscript{64} Fluorescence quenching is classified to two mechanisms: static quenching and dynamic quenching. In the static mechanism, the fluorophore and the quencher collide together in the ground state whereas in dynamic mechanism, fluorophore and quencher collide together in the excited state.\textsuperscript{65} Linearity of the Stern-Volmer plot revealed that quenching fluorescence has only one mechanism, dynamic or static. The observed \(k_q\) values (22.38-34.84 \(\times\) \(10^{11}\) M\(^{-1}\) s\(^{-1}\)), for complexes (1-4) were more than that of limiting diffusion constant (\(K_{\text{diff}}\) of the biomolecules = 2 \(\times\) \(10^{10}\) mol\(^{-1}\) s\(^{-1}\)) which corroborate well with the static quenching (Table 5.4).

\subsection*{5.4.8.6.1 Analysis of binding parameters}

For static quenching mechanism, to evaluate the binding sites (n) and binding constant (\(K_b\)), modified Stern-Volmer equation (5.8) was used.\textsuperscript{66}

\[\log \left(\frac{F_0}{F-1}\right) = \log K_b + n \log [Q] \quad \text{(5.8)}\]

The binding parameters (\(K_b\) = binding constant and \(n\) = number of binding sites) for the complexes were determined via intercepts and slopes, respectively, using linear fitting plots of \(\log (F_0-F)/F\) versus \(\log [\text{complex}]\) as displayed in Figure 5.13(A3) and the values are given in Table 5.4.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{modified_stern_volmer_plot.png}
\caption{Modified Stern-Volmer plot (a-d) for HSA-in presence of complexes (1-4).}
\end{figure}
Table 5.4 The Stern-Volmer constant (K_{SV}), the bimolecular rate constant of HSA (k_{q}), HSA-binding constant (K_{b}) and the number of binding site (n) of the complexes (1-4).

<table>
<thead>
<tr>
<th>Complexes</th>
<th>$K_{sv}$ ($\times 10^3$ M$^{-1}$)</th>
<th>$k_{q}$ ($\times 10^{11}$ M$^{-1}$s$^{-1}$)</th>
<th>$K_{b}$ ($\times 10^3$ M$^{-1}$)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>22.38</td>
<td>22.38</td>
<td>38.99</td>
<td>1.30</td>
</tr>
<tr>
<td>(2)</td>
<td>28.64</td>
<td>28.64</td>
<td>35.71</td>
<td>1.24</td>
</tr>
<tr>
<td>(3)</td>
<td>34.84</td>
<td>34.84</td>
<td>53.54</td>
<td>1.26</td>
</tr>
<tr>
<td>(4)</td>
<td>25.96</td>
<td>25.96</td>
<td>21.79</td>
<td>1.22</td>
</tr>
</tbody>
</table>

For the complexes (1-4), the observed binding constant values were found to be in the range of (21.79-53.54) $\times 10^4$ M$^{-1}$ consistent with the range for optimum $K_{b}$ values ($10^4$-10$^6$ M$^{-1}$), adequate for drug carriers in blood. The results suggested that the $K_{b}$ values of complexes (1-4) to HSA were notably below the association constant of known non-covalent bonds of avidin-ligands interaction ($K \sim 10^{15}$ M$^{-1}$). This suggested a probable release from the serum albumin to the targeted cancer cells, as very high binding constant between a compound and the albumins creates obstruction in the compound release.$^{67,68}$

5.4.8.7 Energy transfer between complexes with HSA

FRET interaction is a distance dependent contact where excitation energy is transferred non-radiatively from a donor to an acceptor molecule. Figure 5.14 display the overlapping of the absorption spectrum of complexes (1-4) with the HSA fluorescence emission spectrum. The values of $E_{FRET}$, $J$, $R_0$ and $r$ were calculated on the basis of aforementioned equations (5.2-5.4) and given in Table 5.5. The distances of donor to acceptor ($r$) were observed to be lower than 7 nm indicating the possibility of transfer of non-radiation energy from HSA to complex.$^{69}$ However, critical distance ($R_0$) of complexes (1-4) is lesser than that of respective $r$, signifying the possibility of static quenching mechanism between the HSA and complex.$^{70}$
Figure 5.14 (a-d) Spectral overlaps of absorption spectra of complexes (1-4) (green) with fluorescence of HSA (orange). Concentration of HSA and complexes were fixed at 2 μM for both HSA and complexes.

Table 5.5 Different energy transfer parameters obtained from overlapping spectra of fluorescence emission and UV absorption.

<table>
<thead>
<tr>
<th>Complexes</th>
<th>E (× 10^{-15} cm^3 L mol^{-1})</th>
<th>J (nm)</th>
<th>R_0 (nm)</th>
<th>r (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>0.141</td>
<td>4.37</td>
<td>2.14</td>
<td>2.84</td>
</tr>
<tr>
<td>(2)</td>
<td>0.150</td>
<td>4.07</td>
<td>2.11</td>
<td>2.80</td>
</tr>
<tr>
<td>(3)</td>
<td>0.139</td>
<td>5.30</td>
<td>2.21</td>
<td>2.93</td>
</tr>
<tr>
<td>(4)</td>
<td>0.155</td>
<td>3.18</td>
<td>2.03</td>
<td>2.69</td>
</tr>
</tbody>
</table>

5.4.8.8 Secondary structure analysis by CD
To ascertain the possible effect of the complex binding on HSA secondary structure, CD measurement was performed in absence and presence of the complexes at different concentrations. In view of the fact that CD spectra of HSA display two negative bands at 208 and 222 nm (UV region) assignable to Π→Π* and n→Π*
transfers for the peptide bond, respectively, which are the characteristic to the protein alpha helical structure\(^1\) (Figure 5.15).

![Figure 5.15 (a-d) Far-UV CD spectra of HSA alone and in the presence of complexes (1-4) at ratios of 1:0, 1:2, 1:4 and 1:6.]

With increasing complexes concentration, the CD signal of HSA leads to increase in both negative minima peaks i.e., 208 and 222 nm without any substantial shift of the peaks. This suggests that the binding of complexes (1-4) to HSA induces an increase in the HSA \(\alpha\)-helical content. However, the CD spectra of HSA were similar in shape in the presence or absence of the complexes revealing that the HSA structure was also predominately \(\alpha\)-helix even on the addition of the complexes.\(^2\) The contents of the \(\alpha\)-helix in free HSA and the complex-HSA can be calculated using following equation\(^3\) and the results were expressed as the mean residual ellipticity (MRE) in deg cm\(^2\) dmol\(^{-1}\).

\[
MRE = \frac{\Theta_{\text{obs}}}{10\times n \times c \times l} \quad \text{(5.9)}
\]

where, \(\Theta_{\text{obs}}\) denotes the observed ellipticity in millidegrees, \(C\) and \(l\) denote the molar concentration of protein and path length of cuvette (0.1 cm), respectively while \(n\) is
the number of amino acid residues that is 585 for HSA. The α-helical contents of free and combined HSA can be evaluated from MRE values using the following equation at 222 nm as described by Chen et al.⁷⁴

\[
\% \alpha-helix = \frac{MRE_{222} - MRE_{3030}}{3030} \times 100
\]

where, MRE\textsubscript{222} is the observed mean residue ellipticity value at 222 nm.

The calculated secondary structure content of HSA showed that on addition of complex, helicity of HSA increased in the range of 61.89-69.78% \textit{(Table 5.6)}.

\textbf{Table 5.6 Secondary structural (% α-helix) analysis for the native HSA with all the metal complexes (1-4) where HSA for all the complexes are same (5 μM).}

<table>
<thead>
<tr>
<th>Complexes</th>
<th>HSA (μM)</th>
<th>HSA + Complex (μM)</th>
<th>α-helix (%) at 298 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>05</td>
<td>00</td>
<td>57.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>61.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>66.81</td>
</tr>
<tr>
<td>(2)</td>
<td>05</td>
<td>00</td>
<td>57.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>62.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>63.87</td>
</tr>
<tr>
<td>(3)</td>
<td>05</td>
<td>00</td>
<td>58.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>64.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>69.78</td>
</tr>
<tr>
<td>(4)</td>
<td>05</td>
<td>00</td>
<td>52.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>55.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>61.89</td>
</tr>
</tbody>
</table>

The increment in the HSA α-helical content indicated that the complexes binding to HSA caused some conformational changes however the HSA secondary structure remained predominantly as α-helix. Hence, CD spectra clearly revealed that the complex interaction with HSA tempted modification in secondary structure. The binding propensity of complexes with HSA was in agreement with other biophysical probes.⁷⁵
5.4.8.9 Molecular docking studies on the interaction mechanism of complexes with HSA

Molecular docking is an important computational tool to determine the mode of interaction and amino acid residues involved in binding of compounds to HSA. The docking results displayed in Figure 5.16 and Table 5.7 revealed suitable structural basis to describe the efficient fluorescence quenching mechanism of HSA in complexes (1-4) via hydrophobic interaction.
Figure 5.16 A-D represents best conformation of compounds (1-4) docked to HSA, respectively, and E-H cartoon representation of residues involved in binding of compounds (1-4) to HSA, respectively.

Further, the optimum energy ranked results of the model between complex and protein are mentioned in Table 5.7 where the magnitude of molecular docking binding interaction energy is not same as from fluorescence; this incongruity in the interaction energy may be due to the exclusion of the solvent molecule and rigidity of some other receptor during docking experiment.
Table 5.7 Molecular docking results for the interaction of complexes (1-4) with HSA.

<table>
<thead>
<tr>
<th>Complexes</th>
<th>Amino acid residues</th>
<th>Nature of interaction</th>
<th>Energy (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>Phe163- Asp162- His139- Val138- Leu147- Phe31- Glu33- Thr28- Gly305- Ala306- Trp303- Glu102- Leu103- Glu145- Ser161</td>
<td>hydrophobic</td>
<td>-4.26</td>
</tr>
<tr>
<td>(2)</td>
<td>Asp162- Ser161- Val34- Gln305- Ileu160- Leu148- Leu142- Phe163- Lys143- Gly29</td>
<td>hydrophobic</td>
<td>-4.38</td>
</tr>
<tr>
<td>(3)</td>
<td>Ser161- Asp162- Val34- Glu33- Leu148- Pro144- Gly305- Ala31- Phe163- Asn146</td>
<td>hydrophobic</td>
<td>-4.20</td>
</tr>
<tr>
<td>(4)</td>
<td>Arg140- His139- Ileu137- Gly136- Val138- Tyr195- Pro194- Phe163- Lys197- Glu66- His133- Leu132</td>
<td>hydrophobic</td>
<td>-4.17</td>
</tr>
</tbody>
</table>

5.5 CONCLUSION

The bio-efficient homobinuclear macrocyclic metal complexes were synthesized with 3,3'-diaminobenzidine, 1,2-bis(bromomethyl)benzene and ethane-1,2-diamine. These were investigated from several spectroscopic and physicochemical techniques which confirmed their formation, stoichiometry and bonding nature. The synthesized complexes exhibit potent antibacterial activity and the DPPH radical scavenging property results proved that these complexes exhibited suitable scavenging potential. In addition, fluorescence spectroscopy displayed an important quantitative data for the binding profile of complexes with HSA which showed that metal complexes quench the Trp of HSA fluorescence following the order of 3>1>2>4 with high binding
affinity with HSA. Furthermore, the molecular docking ascertained possible binding modes and deciphered that hydrophobic forces have key role in the binding of complexes to HSA. This study may provide significant platform in the understanding of the complexes interaction with HSA and thus exhibited potent role in the biomedical and pharmaceutical application.


5.6 REFERENCES


