CHAPTER III

β-cyclodextrin based Schiff base Zn(II) complexes: Synthesis, Physicochemical Characterization and their role in alleviating oxidative stress related disorder*

3.1. Introduction

Certain inorganic compounds play crucial roles in biological processes and many organic compounds used as medicine are activated by metal ions.[1] Schiff bases, their derivatives and their metal complexes are often active as anticancer and antioxidative agents.[2-4] Transition metal ions readily complexes with polydentate Schiff base ligands specially containing nitrogen, oxygen or sulphur donor atoms and they have great importance in biological systems.[5,6] In biological oxygen carrier systems such complexes may act as model molecules.[7] For example, Zishen *et al.*[8] have reported that Schiff base complexes derived from 4-hydroxysalicylaldehyde and amines showed strong anticancer activities against *Ehrlich ascites carcinoma* (EAC). However, poor aqueous solubility of such complexes often poses a problem for their applications in biochemical activities. Carbohydrates have high aqueous solubility and many antibiotics containing sugar or carbohydrate moieties readily interact with DNA molecules.[9]

Although oxygen is crucial for the survival of living organism but most of the unused oxygen transforms into various reactive oxygen species (ROS), which generate oxidative stress in the biological systems. Free radicals or reactive oxygen species (ROS) (*i.e*, oxidant) are highly detrimental to the immune systems and often triggers carcinogenic activities through oxidative stress. It damages normal biochemical activities in the living organism,[10] so to combat this foul species various antioxidants must be developed in the organism. During endogenous metabolic reaction aerobic cell produces ROS such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). Also mitochondrial respiratory chain under hypoxic condition generates nitric oxide (NO) which in turn forms reactive nitrogen species (RNS).[11]Thus an optimum level ROS is essential for normal biological functions like cellular growth, gene expression and defence against infection.[12] On the contrary, nitric oxide (NO) is formed from the arginine and aided by nitric oxide synthase (NOS). Peroxynitrate is a resultant of the reaction between

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nitric oxide and superoxide, a potent and very active oxidant that can attack a wide array of biological functions.

Therefore, herein this chapter two *β*-cyclodextrin based Schiff bases (3a and 3b) have been synthesized and used to prepare water soluble Zn(II) complexes (4a and 4b), their potential effects in alleviating oxidative disorder or oxidative stress were studied through various biochemical methods in order to evaluate the free radical scavenging activities of the synthesized Zn(II) complexes (4a and 4b).

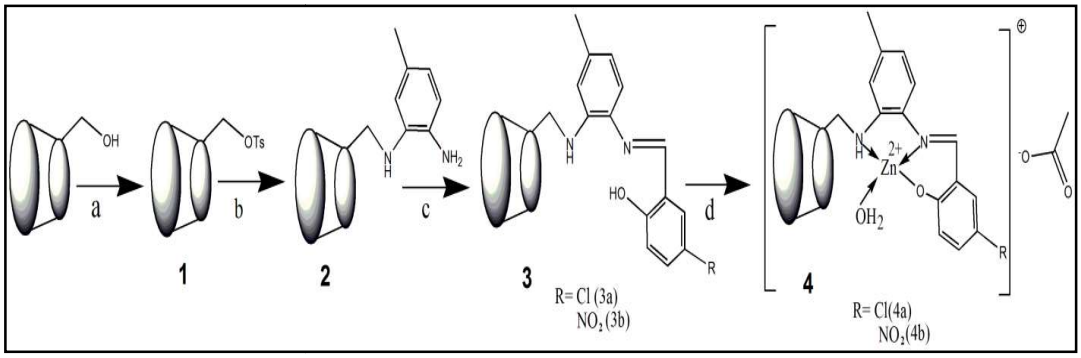
3.2. Experimental section

3.2.1. Materials and methods

All the chemicals were purchased from Sigma Aldrich, Germany and were used without further purification. Bi-distilled water was used in all the experiments. FTIR spectra were recorded on Perkin-Elmer Spectrum FTIR spectrometer (RX-1) using KBr pellets in the range 4000-400 cm^{-1} at ambient temperature and UV-Visible spectra were recorded on a JascoV-530 double beam Spectrophotometer using quartz cell (1 cm) equipped with thermostated bath (maintained at 25±0.1 °C) using water and DMSO as solvent reference. ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature on a Bruker Advance-II 400 MHz spectrometer by using D_2O and DMSO-d_6 as solvents and chemical shifts (δ) were quoted in ppm with respect to TMS. Elemental microanalyses (C, H and N) were conducted by using Euro VECTOR EA 3000 analyzer. Zn-content was determined by AAS (Varian, SpectraAA 50B) by using standard Zn-solution from Sigma-Aldrich, Germany. The purity of the ligands and its Zn(II) complexes were confirmed by TLC. The mass spectra were recorded by using Waters ZQ-4000 instrument. All the experiments using animals were reviewed and approved by the Animal Ethical Committee of University of North Bengal (Permit No. 840/ac/04/CPCSEA, Committee for the Purpose of Control and Supervision on Experiments on Animals) and performed in accordance with the legislation for the protection of animals used for scientific purposes. Blood were collected from Swiss albino mice by puncturing the heart under proper anesthesia. Collected blood samples were used for erythrocyte membrane stabilizing and hemolytic activities. Brain was isolated from the same mouse for lipid peroxidation assay. The detailed description of the various analytical and spectroscopic methods and instruments has already been discussed in chapter II.

3.2.2. Synthesis

The synthetic path of *β*-cyclodextrin based Schiff bases and their Zn(II) complexes are shown in scheme 3.1.



Scheme 3.1. Syntheses of compound 1, 2, 3 (3a and 3b) and 4 (4a & 4b).

3.2.2.1. Synthesis of mono-6-deoxy-6-(*p*-tosylsulfonyl)-*β*-cyclodextrin [*β*-CDOTs, 1]

p-toluenesulfonyl chloride (2.5 g, 1.5 equiv.) was added dropwise to a round bottom flask containing *β*-cyclodextrin (*β*-CD) (10.0 g, 8.8 mmol) dissolved in pyridine (500 mL) and stirred over night at room temperature. After 24 hours the solution gave three components as observed by TLC (SiO_2; butanol:ethanol:water = 5:4:3 (vol.) and the reaction was ceased by adding 10 mL water. Then the reaction mixture was concentrated to one fifth in volume and added acetone dropwise until the precipitation was over. The precipitate was filtered, washed with acetone and was dried under vacuum. The precipitate was dissolved in DMF and purified on a reversed-phase column (silica gel 60 silanized, 200 g in dry weight, 4.1 × 30 cm), by using 5-40 % DMF (aq) as eluent. The fraction of 5-10 % contained *β*-CD while TsO-*β*-CD was in 10-20 % and 20-40 % contained $(TsO)_2$-*β*-CD.Then the fraction of 10-20 % was concentrated and was poured into acetone to obtain white coloured pure product. The product was filtered, dried and stored in a vacuum desiccator.

Color: white; Yield (45%); IR cm^{-1}, KBr: 3391 (OH), 2926 (C–H), 1646 (C=C), 1366 (SO_2 Assym), 1153 (SO_2 Sym) cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6, 25 °C): δ = 7.49 (m, 2H Ph), 7.12 (m, 2H Ph), 5.94–5.72 (m, 14H, OH2 and OH3 CD), 4.87–4.76 (m, 7H, H1 CD), 4.58–4.43 (m, 6H, OH6 CD), 4.27 (m, 2H, H6/ CD), 4.02 (m, 1H, H5/ CD), 3.92–3.54 (m, 25H, H3, H5 and H6 CD), 3.47–3.14 (m, H2, H4 overlap with water), 2.28 (s, 3H, Ph-CH_3) ppm;[13] ^{13}C NMR (DMSO-d_6): δ = 146.52,

143.15, 127.91, 124.26 (aromatic), 101.84 (C1), 81.06 (C4), 73.03,72.06, 71.68 (C3,C2,C5), 62.88 (C6$^{/}$), 60.16 (C6), 23.18(1 (C9) ppm;[14] Anal. Calcd. for $C_{49}H_{76}O_{37}S$: C, 45.65; H, 5.94; O, 45.92. Found: C, 45.05; H, 5.29; O, 45.53.

3.2.2.2. Synthesis of mono-6-deoxy-6-(toluene-3,4-diamino)-*β*-cyclodextrin (2)

In a 50 mL round bottom flask 3,4-diaminotoluene (85.3mg, 0.7826 mmol) was dissolved in dry DMF (2.0 mL) and warmed to 50 °C. Dimethylaminopyridine (DMAP) (97.0 mg, 0,795 mmol) and KI (32.2 mg, 0.194 mmol) were added, and after 5 min powdered *β* -CDOTs (1) (500 mg, 0.388 mmol) was added. The reaction was stirred for 36 h at 50 °C. The reaction was cooled to room temperature and the volatile materials were removed in vacuo to give a yellow mass. The crude product was dissolved in water and reprecipitated out by adding acetone to it. The precipitate was collected by filtration and after drying solid product was obtained.

Color: Yellowish white; Yield (63%); IR, KBr cm^{-1}: 1641, 1557, 1412 and 1261 cm^{-1}; UV: 255 nm, 216 nm; ^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 7.18 (m, 1H, Ph), 7.09 (m, 1H, Ph), 6.71 (m, 1H, Ph), 5.82–5.67 (m, 14H, OH2 and OH3 CD), 5.02 (s, 1H, NH), 4.84-4.75 (m, 7H, H1 CD), 4.65 (s, 2H, NH_2), 4.51–4.40 (m, 6H, OH6 CD), 4.24 (m, 2H, H6$^{/}$ CD), 4.08 (m, 1H, H5$^{/}$ CD), 3.93–3.80 (m, 25H, H3, H5 and H6 CD), 3.62–3.51 (m, H2, H4 overlap with water), 2.67 (s, 3H, -CH_3) ppm; ^{13}C NMR (D_2O): δ = 134.21, 130.70, 116.81, 117.96, 113.35 (aromatic), 101.66 (C1), 81.17 (C4), 73.07,72.91, 71.64 (C3,C2,C5), 61.12 (C6), 40.06 (C6$^{/}$), 24.31(1 (Cf) ppm; Anal. Calcd for $C_{49}H_{77}N_2O_{34}$: C, 47.53; H, 6.27; N, 2.26; O, 43.94. Found: C, 47.08; H, 6.19; N, 2.04; O, 43.35

3.2.2.3. General procedure for synthesis of amino modified *β*-cyclodextrin based Schiff bases (3a & 3b)

A mixture containing amino modified *β*-cyclodextrin (2) and aldehyde (a, b) in 50 mL1:1 (v/v) aqueous ethanol was refluxed for 4 hours at 60 °C. After the completion of reaction, the water soluble solid mass was obtained by complete evaporation of the solvent in hot water bath. The product was recrystallized several times from aqueous ethanol to remove impurity and ultimately pure solid products were obtained.

Physical and spectral data for 3a and 3b are as follows:

3.2.2.3.1. Mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylidene amino) - 3,4-diaminotolune)-*β*-cyclodextrin (3a)

Color: Yellowish white; Yield (66%); IR cm^{-1}: 1636, 1337, 1263 ; ^1H NMR (400 MHz, D_2O, 25 °C): δ = 10.12 (s, 1H, Phenolic -OH), 8.18 (s, 1H, CH=N), 6.58 – 7.45 (m, aromatic protons), 5.87–5.69 (m, 14H, OH2 and OH3 CD), 5.28 (s, 1H, NH), 4.91–4.83 (m, 7H, H1 CD), 4.58–4.43 (m, 6H, OH6 CD), 4.32 (m, 2H, H6/ CD), 4.10 (m, 1H, H5/ CD), 3.94–3.80 (m, 25H, H3, H5 and H6 CD), 3.62–3.51 (m, H2, H4 overlap with water), 2.18 (s, 3H, $-CH_3$) ppm; ^{13}C NMR (D_2O): δ = 160.08 (-C-OH), 158.62 (-C=CN), 144.20, 136.44, 135.80, 132.92, 130.70,127.43, 122.07, 119.57, 118.84, 117.19, 112.65 (aromatic), 101.66 (C1), 81.17 (C4), 73.17,72.93, 71.57 (C3,C2,C5), 61.15 (C6), 44.69 (C6/), 24.16(Cf) ppm; UV: 256 nm, 282 nm; Anal. Calcd for $C_{56}H_{80}N_2O_{35}Cl$: C, 48.85; H, 5.86; N, 2.03; O, 40.66. Found: C, 48.68; H, 5.29; N, 1.89; O, 40.24. m/z (ESI): calculated 1376.64, found 1377.32 $[M+H]^+$.

3.2.2.3.2. Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylidene amino) - 3,4-diaminotolune)-β-cyclodextrin (3b)

Color: Pale yellow ; Yield (69%); IR cm^{-1}, KBr: 1628, 1340, 1263; ^1H NMR (400 MHz, D_2O, 25 °C): δ = 10.11 (s, 1H, Phenolic -OH), 8.12 (s, 1H, CH=N), 6.85 – 7.69 (m, aromatic protons), 5.89–5.72 (m, 14H, OH2 and OH3 CD), 5.16 (s, 1H, NH), 4.90–4.78 (m, 7H, H1CD), 4.38–4.55 (m, 6H, OH6 CD), 4.31 (m, 2H, H6/ CD), 4.12 (m, 1H, H5/ CD), 3.93–3.80 (m, 25H, H3, H5 and H6 CD), 3.60–3.51 (m, H2, H4 overlap with water), 2.18 (s, 3H, $-CH_3$) ppm; ^{13}C NMR (D_2O): δ = 164.53 (-C-OH), 160.14 (-C=CN), 146.43, 140.56, 137.70, 135.41, 125.48, 124.80, 123.12, 119.33, 118.83, 116.90, 114.56 (aromatic), 101.78 (C1), 81.04 (C4), 73.14, 72.84, 71.60 (C3,C2,C5), 60.47 (C6), 46.51 (C6/), 23.95(Cf) ppm; UV: 250 nm, 284 nm; Anal. Calcd for $C_{56}H_{80}N_3O_{37}$: C, 48.48; H, 5.81; N, 3.03; O, 42.67. Found: C, 48.21; H, 5.19; N, 2.81; O, 42.38. m/z (ESI): calculated 1387.23, found 1388.53 $[M+H]^+$.

3.2.2.4. General procedure for synthesis of Zn(II)-metal complexes of amino modified *β*-cyclodextrin based Schiff bases (4a & 4b)

The amino modified *β*-cyclodextrin based Schiff bases 3a (275.3 mg ,0.2 mmol) & 3b (277.4 mg, 0.2 mmol) respectively were stirred with aqueous methanol for 10 min. Zinc acetate (43.9 mg, 0.2 mmol) was added to the resulting solutions and the mixture were refluxed for 6 h. Then the reaction mixtures were concentrated

and on addition acetone the product was precipitate out. The products were purified from water-methanol (1:1v/v) several times.

Physical and spectral data for 4a and 4b are as follows:

3.2.2.4.1. Zn(II) complex of mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-*β*-cyclodextrin (4a)

Color: Light yellow; Yield (75%); IR cm^{-1}, KBr: 1570, 1330, 1244, 859, 775, 525, 440; ^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 8.39 (s, 1H, CH=N), 6.45 – 7.32 (m, aromatic protons), 6.12–5.97 (m, 14H, OH2 and OH3 CD), 5.61 (s, 1H, NH), 4.93–5.08 (m, 7H, H1 CD), 4.72–4.60 (m, 6H, OH6 CD), 4.45 (m, 2H, H6$^{/}$ CD), 4.15 (m, 1H, H5$^{/}$ CD), 3.93–3.80 (m, 25H, H3, H5 and H6 CD), 3.61–3.51 (m, H2, H4 overlap with water), 1.86 (s, 3H, -CH_3) ppm; ^{13}C NMR (D_2O): δ = 169.84 (-CO of oAc^-), 157.98 (-C-OH), 155.13 (-C=CN), 143.36, 137.12, 135.56, 131.87, 130.17,127.52, 122.12, 119.87, 118.61, 117.16, 112.31 (aromatic), 101.66 (C1), 81.17 (C4), 73.17,72.93, 71.57 (C5,C2,C3), 61.15 (C6), 57.43 (C6$^{/}$), 25.26(Cf), 23.21 (-CH_3 of oAc^-) ppm; UV: 282 nm, 261 nm, 366 nm; Anal. Calcd for $C_{58}H_{84}N_2O_{38}$ Cl Zn: C, 45.88; H, 5.58; N, 1.84; O, 40.05; Zn, 4.31. Found: C, 45.58; H, 5.19; N, 1.49; O, 39.85; Zn, 4.11. m/z (ESI): calculated 1518.12, found 1519.35 $[M+H]^+$.

3.2.2.4.2. Zn(II) complex of mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (4b)

Color: Canary yellow; Yield (78%); IR cm^{-1}, KBr: 1582, 1332, 1241, 862, 762, 533, 437; ^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 8.35 (s, 1H, CH=N), 6.98 – 7.83 (m, aromatic protons), 6.03–5.96 (m, 14H, OH2 and OH3 CD), 5.63 (s, 1H, NH), 4.96–5.10 (m, 7H, H1 CD), 4.76–4.67 (m, 6H, OH6 CD), 4.49 (m, 2H, H6$^{/}$ CD), 4.25 (m, 1H, H5$^{/}$ CD), 3.93–3.80 (m, 25H, H3, H5 and H6 CD), 3.61–3.51 (m, H2, H4 overlap with water), 1.86 (s, 3H, -CH_3) ppm;^{13}C NMR (D_2O): δ = 173.16 (-CO of oAc^-), 162.25 (-C-OH), 157.02 (-C=CN), 145.89, 140.92, 138.06, 136.87, 127.49,125.22, 119.32, 119.87, 118.92, 116.09, 114.73 (aromatic), 101.62 (C1), 81.19(C4), 73.12, 72.79, 71.62 (C3,C2,C5), 60.32 (C6), 57.66 (C6$^{/}$), 24.01(Cf), 22.78 (-CH_3 of oAc^-) ppm; UV: 284 nm, 257 nm, 397 nm; Anal. Calcd for $C_{58}H_{84}N_3O_{40}Zn$: C, 45.57; H, 5.53; N, 2.75; O, 41.86; Zn, 4.28. Found: C, 45.08; H, 5.19; N, 2.49; O, 41.25; Zn, 4.06. m/z (ESI): calculated 1528.67, found 1528.96$[M]^+$.

3.2.3. Antioxidant and free radical scavenging activity study

The detailed description of the various experimental methods and instruments for performing the antioxidant and free radical scavenging activity study has been discussed in chapter II (sections 2.5, 2.6, 2.7 and 2.8).

3.3. Results and discussion

Mono-6-deoxy-6-(toluene-3,4-diamino)-*β*-cyclodextrin (2) was synthesized from mono-6-deoxy-6-(*p*-tosylsulfonyl)-*β*-cyclodextrin (*β*-CDOTs) (1). After synthesis of the amino modified *β*-cyclodextrin based Schiff bases (3a & 3b), it was allowed to react with zinc acetate in aqueous methanol to give amino modified *β*-cyclodextrin based zinc complexes (4a & 4b) (Scheme 3.1). The structure of Zn(II) complexes were confirmed by various analytical and spectroscopic analyses, such as elemental analysis, FTIR, ^{1}H NMR, ^{13}C NMR, UV-visible spectroscopy and ESI-MS.

3.3.1. FTIR spectra

The FTIR spectrum of compound 1 and 2 were shown in (Figure 3.1). The characteristic absorption peaks at 1646 (C=C), 1366 (SO_2 Assym), 1153 (SO_2 Sym) cm^{-1} in the IR spectrum of 6-OTs-*β*-CD (1)[15] which corresponding to sulfonic acid group, have disappeared in the IR spectrum of compound 2. On the other hand, four characteristic absorption peaks of 1641, 1557, 1412 and 1261 cm^{-1} appears in the spectrum of compound 2. The absorption peaks of 1641 cm^{-1} was corresponded to N-H stretching vibration of the group of 3,4-diaminotolune moiety. The absorption peaks of 1412 cm^{-1} was corresponded to -CH_2- stretching vibration. The peaks at 1557 and 1261 cm^{-1} were assigened to C=C stretching vibrations for the phenyl moiety of compound 2. The results showed that the *p*-toluenesulfonyl group had been substituted by 3,4-diaminotolune group. In the FTIR spectrum of 4a & 4b (shown in Figure 3.2), the azomethine peak was shifted to lower frequency than the ligands 3a & 3b (shown in Figure 3.3) and appeared at 1570 and 1582 cm^{-1}, respectively indicating that the N atom of the C=N group of the ligands coordinates with the Zn^{2+} ion. The involvement of deprotonated phenolic moiety in complexes, have been confirmed by the shift of ν(C-O) stretching band [observed at 1337, 1263 cm^{-1} and 1340,1263 cm^{-1} in the free ligands (3a and 3b), respectively] to a lower frequency at 1330, 1244 cm^{-1} and 1332, 1241 cm^{-1} in complexes (4a and 4b), respectively.[16] The shift of ν(C-O) band to a lower frequency suggests the weakening of C-O bond and formation of stronger M-O bond. In the IR spectra of both complexes the bands that appeared at

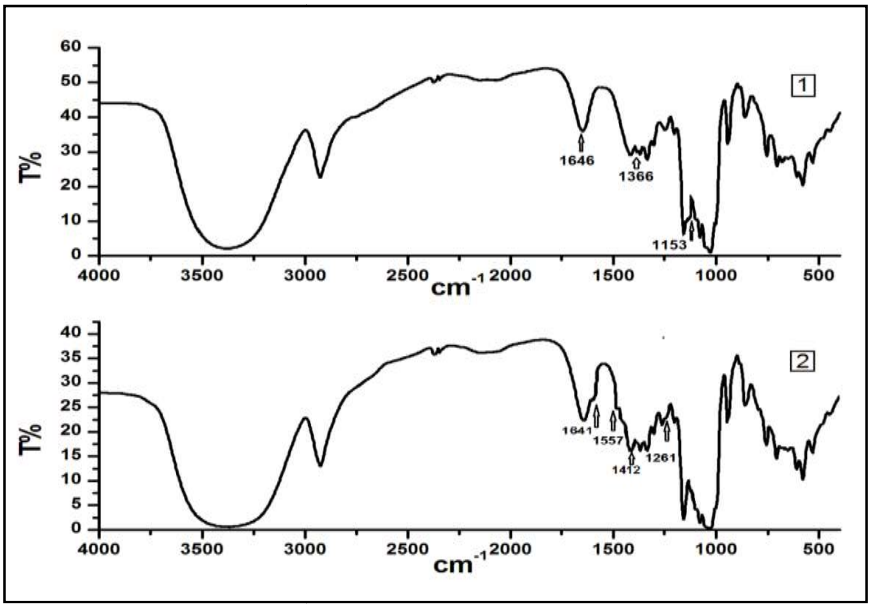


Fig 3.1. FTIR spectra of Mono-6-deoxy-6-(*p*-tosylsulfonyl)-*β*-cyclodextrin [*β*-CDOTs, **1**] and Mono-6-deoxy-6-(toluene-3,4-diamino)-*β*-cyclodextrin (**2**).

862-859 cm^{-1} and 790-760 cm^{-1} were assigned for the rocking and wagging vibration of coordinated water molecule.[17-19]The bands observed at 440 and 437 cm^{-1} were attributed to ν(M-phenolic O) and the band observed at 525 and 533cm^{-1} were attributed to ν(M-N) vibrations for complexes (4a and 4b), respectively.[20]

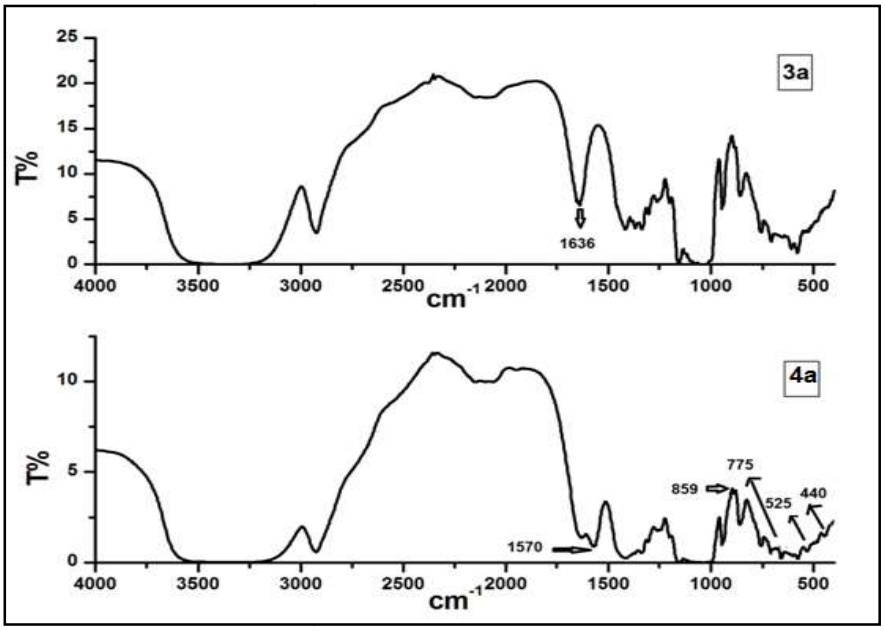


Fig 3.2. FTIR spectra of Mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-*β*-cyclodextrin (**3a**) and Zn complex of Mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-*β*-cyclodextrin (**4a**).

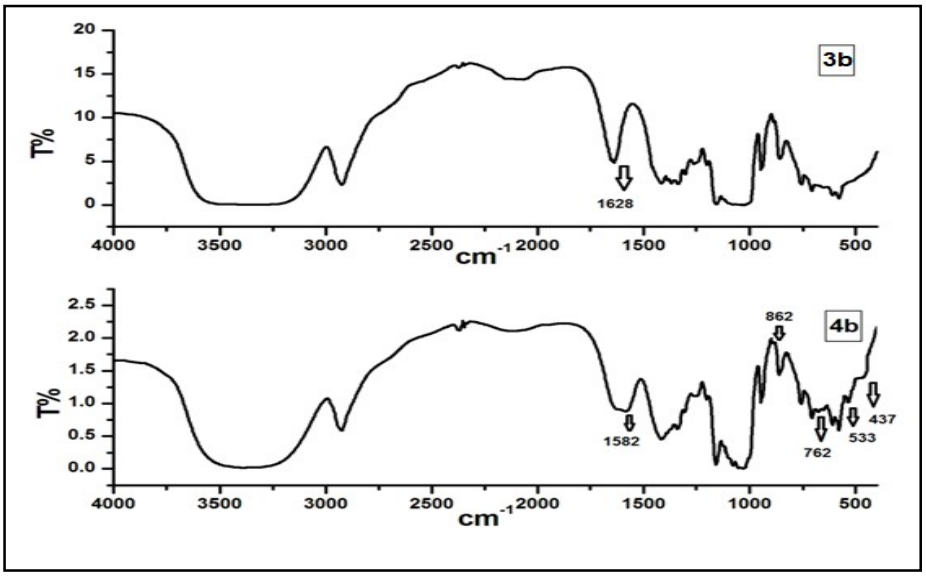


Fig 3.3. FTIR spectra of Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-*β*-cyclodextrin (**3b**) and Zn complex of Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-*β*-cyclodextrin (**4b**).

3.3.2. NMR spectra

^{1}H NMR spectra of the mono-tosyleted *β*-cyclodextrin (1), *β*-cyclodextrin based amine (2), Schiff bases (3a and 3b) and two Zn(II) complexes (4a and 4b) were recorded in D_2O (shown in Figure 3.4 - 3.9). ^{1}H NMR spectrum of compound 2 shows that the peaks at δ ≈ 5.02 ppm for –NH group, at *δ* 2.676 ppm for –CH_3 group. Signals of the aromatic protons appeared as multiplets in the range of *δ* ≈6.7–7.2 ppm and –NH_2 group appeared as a singlet in 4.652 ppm.[21] The signals of the H6 protons with an amino group (H6/ a. H6/ b) appear at 0.8 ppm lower field than those of H6 protons of other rings of *β*-cyclodextrin suggesting the formation of mono-6-deoxy-6-(toluene-3,4-diamino)-*β*-cyclodextrin (2) . After the reaction of amine modified *β*-cyclodextrin with aldehydes the resulting Schiff base ligands (3a and 3b) show the following signals: phenyl protons as multiplets at ≈ 6.8 to 7.9 ppm, -CH_3 protons as singlet at ≈ 2.181-2.0 ppm, the peak at *δ* ≈ 10.0 ppm can be attributated to the phenolic proton present in the salicylaldehyde moiety. Disappear of the singlet of –NH_2 proton suggest formation of azomethine group, azomethine proton (C–CH=N–) peak appears at *δ* ≈ 8.2 ppm in both ligands. Both Zn(II) complexes show that the phenolic –OH group is involved in complexation resulting in the disappearance of the peak at 10.0 ppm. The azomethine proton signal in the spectra of the zinc complexes were shield downfield (near *δ* ≈ 8.40 ppm) compared to the free ligands, suggesting deshielding of the azomethine group due to the coordination with metal ion. The

signals appearing at 5.30 ppm in the spectra of the ligands (due to the proton of –NH group) broadens and appears at $\delta \approx 5.6$ppm in the spectra of the complexes suggesting the coordination of NH group with the metal ion.[22]

The ^{13}C NMR spectra of 1, 2, 3a, 3b, 4a and 4b were recorded in D_2O and DMSO-d_6 and have been shown in Figure 3.10 - 3.15. In ^{13}C-NMR spectrum of compound 2 the signal for carbon (C6$^{/}$) with an amino group appears at around 20 ppm lower field than those of C6 carbon of other rings of *β*-cyclodextrin. This fact confirms the formation of mono-6-deoxy-6-(toluene-3,4-diamino)-*β*-cyclodextrin (2).[14] Both ligands (3a and 3b) showed signals for the –CH=N group at 158.62ppm and 160.14 ppm, respectively. Also these ligands showed ^{13}C signals for –C-OH group at signals at 160.08ppm and 164.53ppm, respectively. The ^{13}C NMR spectra of both ligands (3a and 3b) and complexes (4a and 4b) showed peaks for aromatic carbons at usual range (110-145 ppm). The ^{13}C NMR signals of –CH=N and –C-OH groups in both ligands (3a and 3b) get shifted upfield to 155.13ppm and 157.98ppm for complex 4a and 157.02ppm and 162.25ppm for complex 4b. This upfield shift confirms the coordination of Zn(II) ion with azomethine N atom and phenolic –O atom. ^{13}C NMR signal of C6$^{/}$ in both complexes (4a and 4b) were also shifted to low field (form around 45ppm to around 58ppm) suggesting coordination to Zn(II) ion.[14] Therefore ^{1}H and ^{13}C NMR spectra substantiated the coordination geometry of complexes.

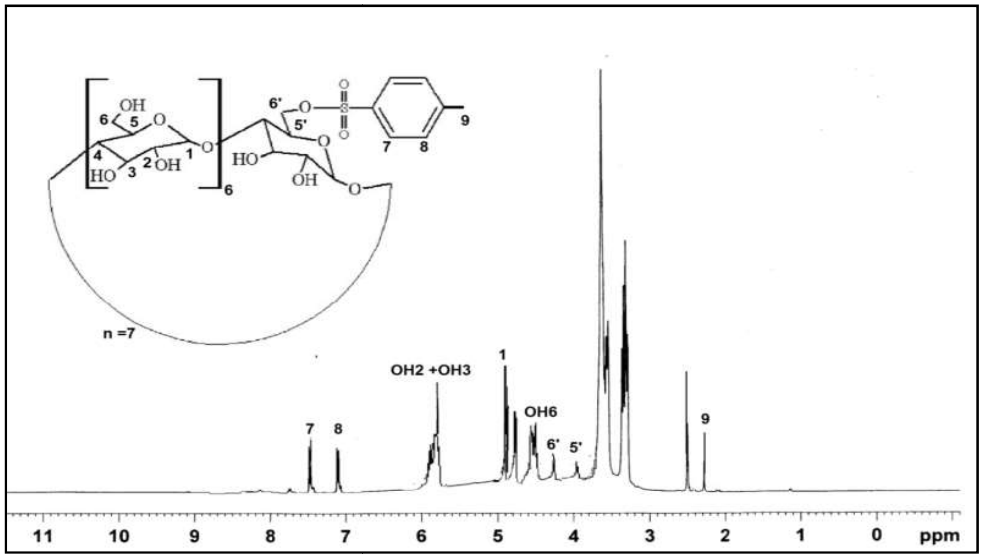


Fig 3.4. ^{1}H NMR of Mono-6-deoxy-6-(*p*-tosylsulfonyl)-*β*-cyclodextrin [*β*-CDOTs, **1**].

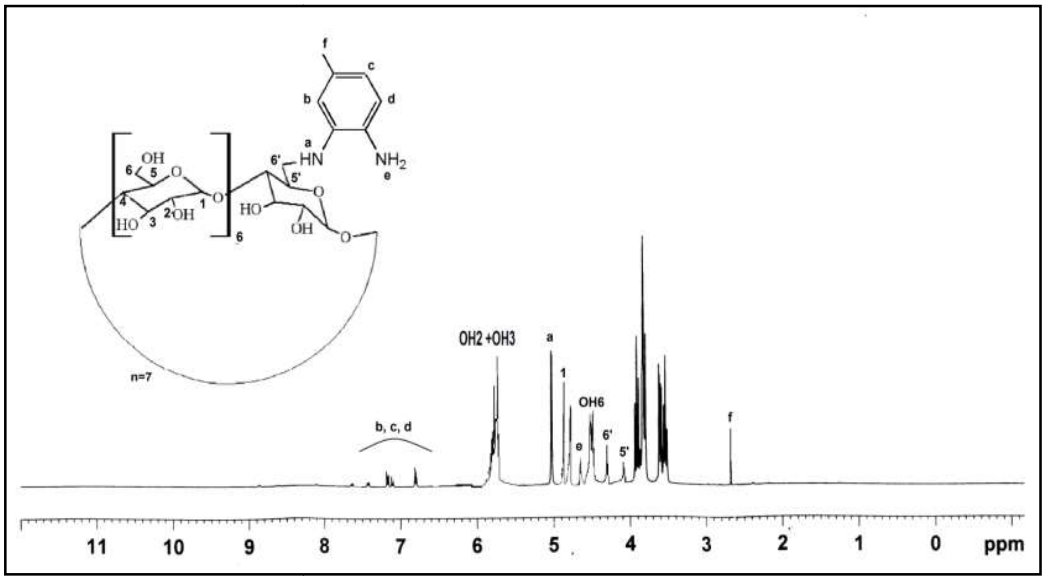


Fig 3.5. [1]H NMR of Mono-6-deoxy-6-(toluene-3,4-diamino)-β-cyclodextrin (**2**).

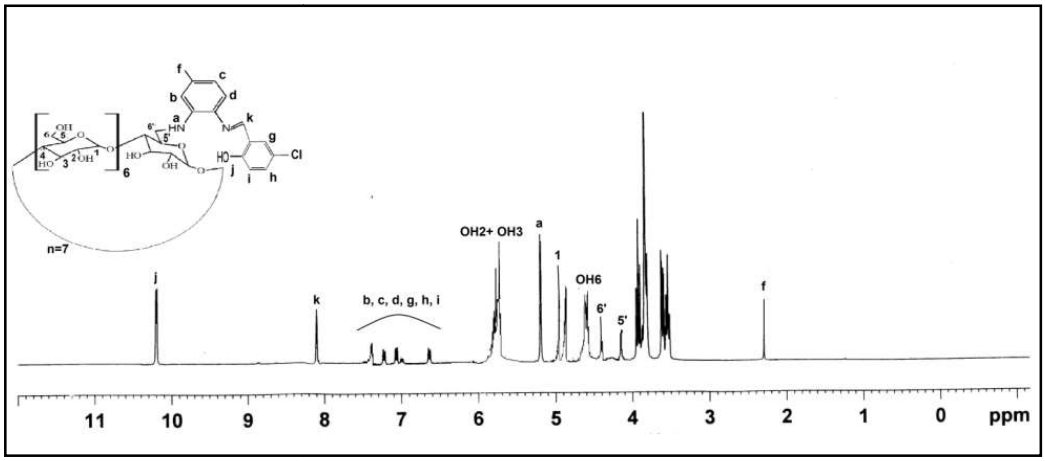


Fig 3.6.[1]H NMR of Mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (**3a**).

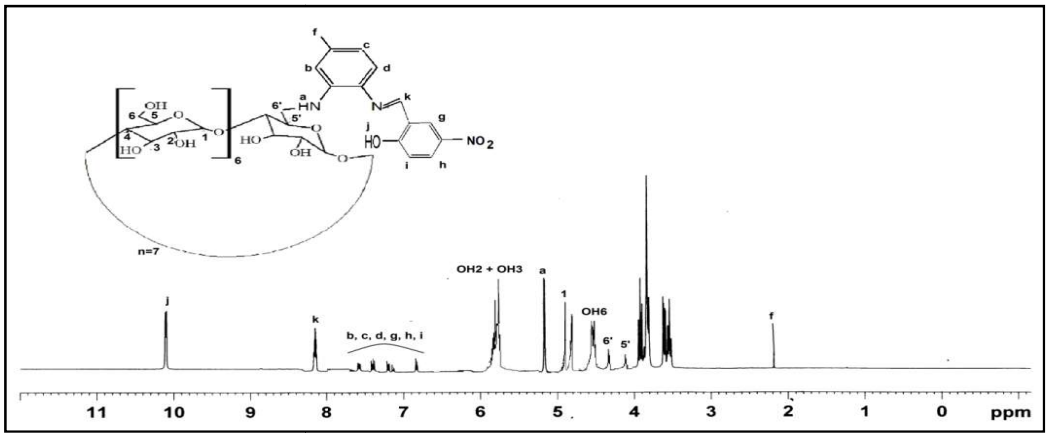


Fig 3.7. [1]H NMR of Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (**3b**).

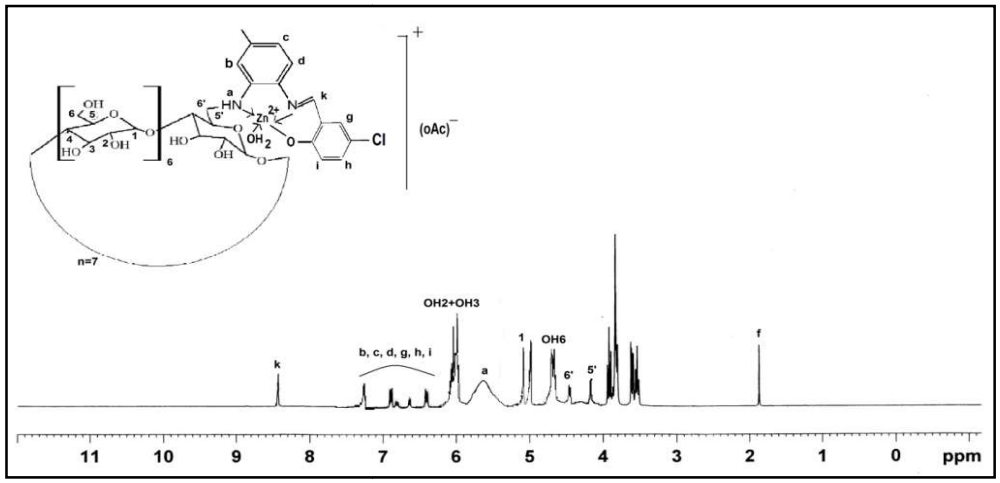


Fig 3.8. ^{1}H NMR of Zn complex of Mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (**4a**).

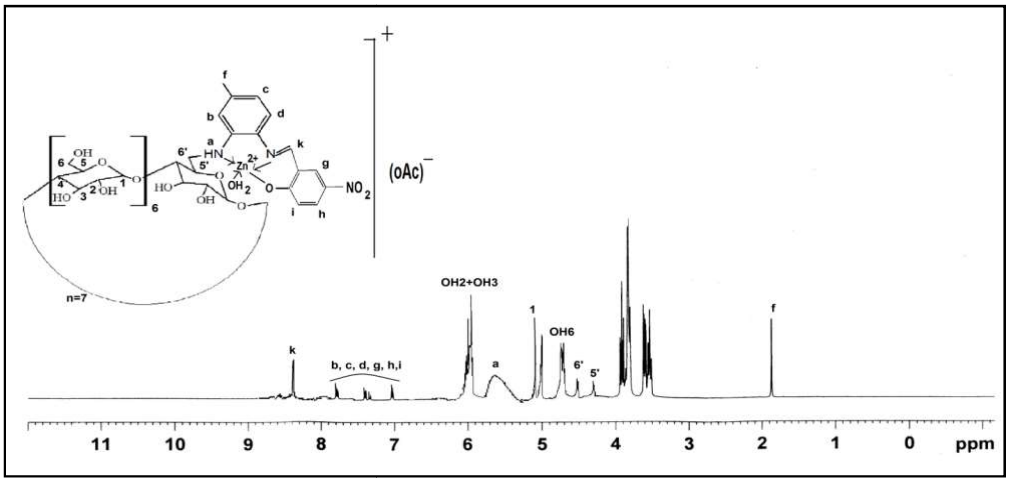


Fig 3.9. ^{1}H NMR of Zn complex of Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (**4b**).

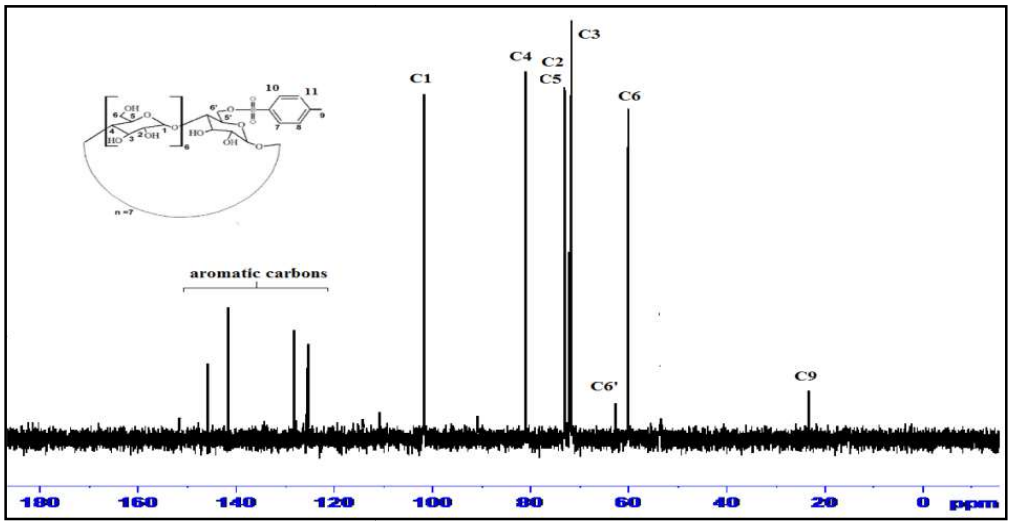


Fig 3.10. ^{13}C NMR of Mono-6-deoxy-6-(p-tosylsulfonyl)-β-cyclodextrin (β-CDOTs) (**1**).

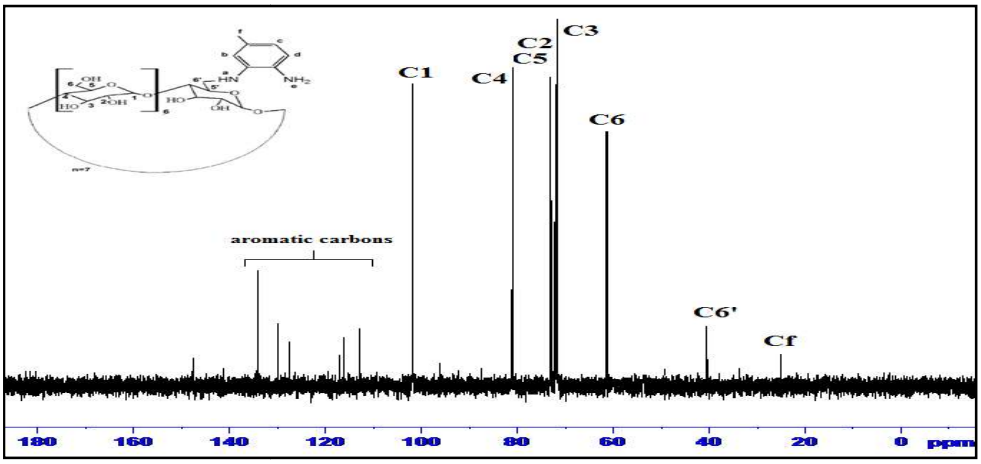


Fig 3.11. ^{13}C NMR of Mono-6-deoxy-6-(toluene-3,4-diamino)-β-cyclodextrin (**2**).

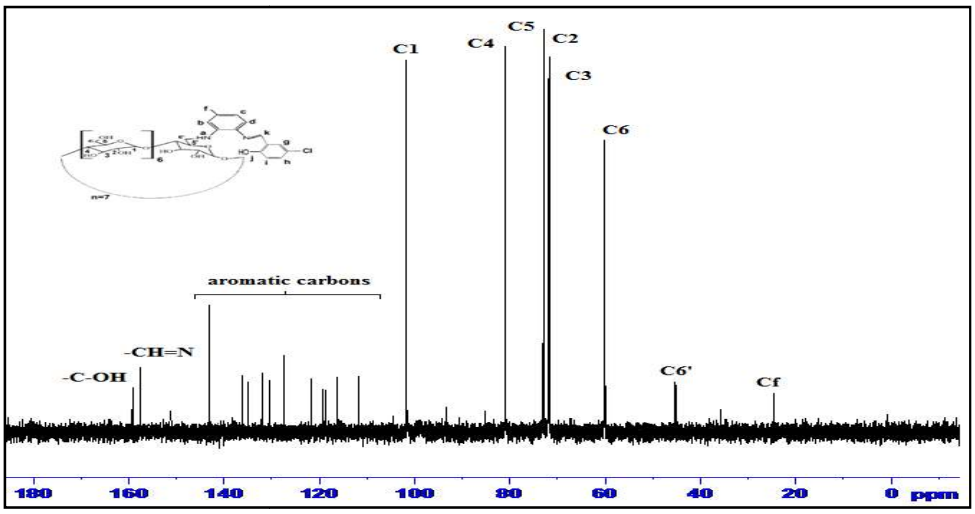


Fig 3.12. ^{13}C NMR of Mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (**3a**).

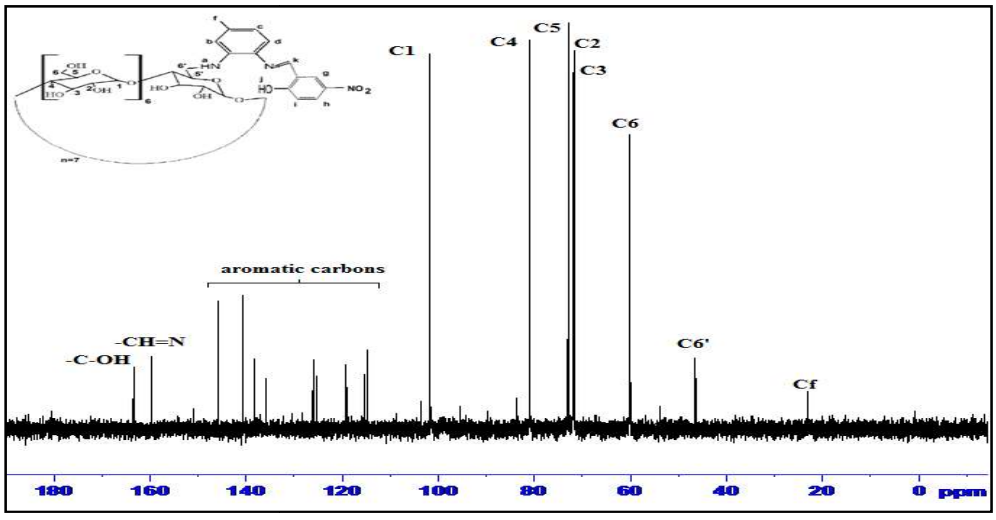


Fig 3.13. ^{13}C NMR of Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (**3b**).

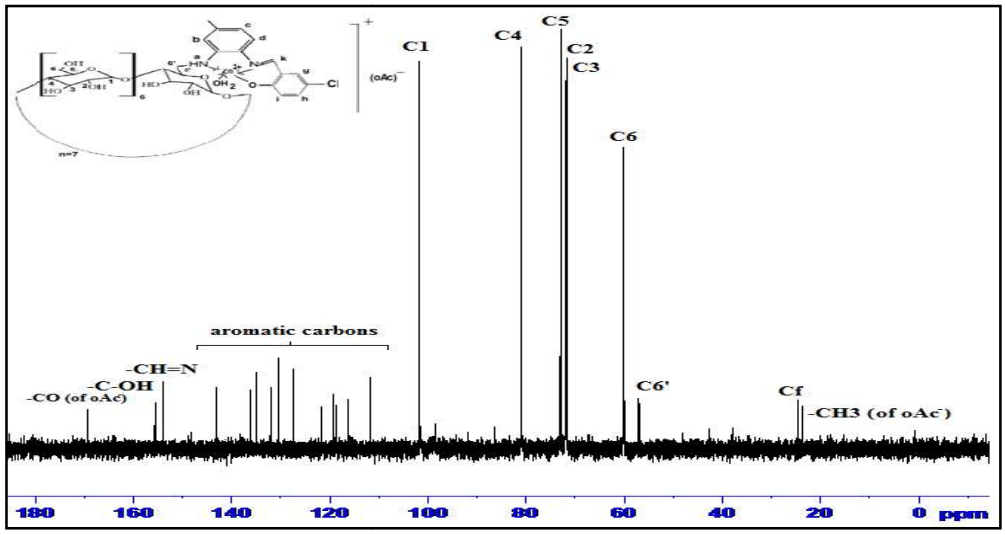


Fig 3.14. ^{13}C NMR of Zn complex of Mono-6-deoxy-6-(4-(5-chloro-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (**4a**).

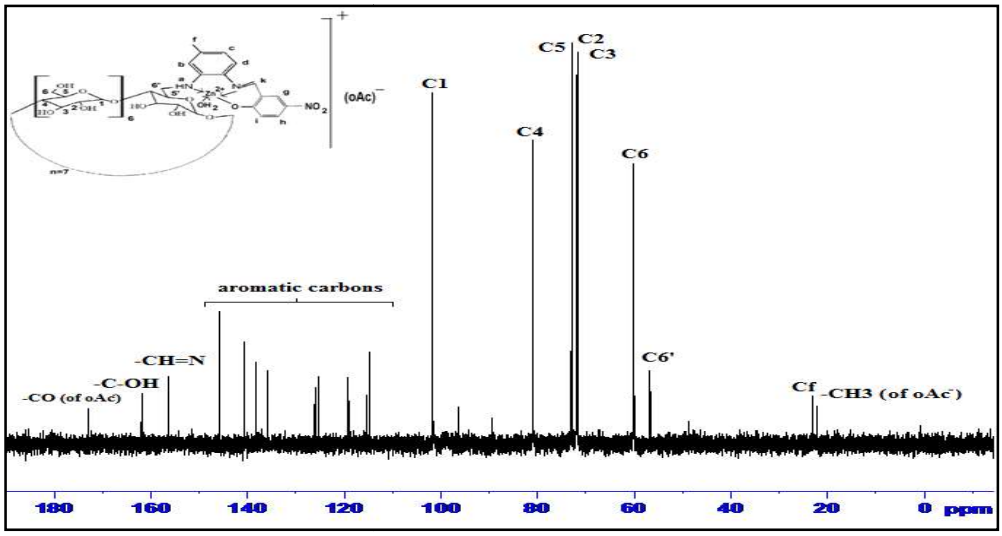


Fig 3.15. ^{13}C NMR of Zn complex of Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (**4b**).

3.3.3. UV-visible spectra

UV-VIS spectra of the β-cyclodextrin based amine (2), Schiff bases (3a and 3b) and two Zn (II) complexes (4a and 4b) were recorded in water (shown in Figures 3.16 -3.18). The modified amine (2) shows two bands at 255 and 216 nm due to the $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ transitions. The electronic spectra of the Schiff base ligands (3a and 3b) exhibit absorption bands at 256 nm & 250 nm, respectively with shoulder at around 280-285 nm which were due to the $\pi\rightarrow\pi^*$ transition.[23] In addition to these

peaks, both compounds showed bands at 311-320 nm attributable to $n \rightarrow \pi^*$ transition.[24,25] In Zn(II) complexes (4a and 4b) the above bands get shifted from 256 nm to 261 nm (bathochromic) and from 250 nm to 257 nm (bathochromic) respectively, suggested the coordination of the metal ion (Zn^{2+}) with ligands. Disappearance of the peaks at 311-322 nm in the complexes compared to that of ligands suggested the coordination of Zn with azomethine N-atom of the ligands.

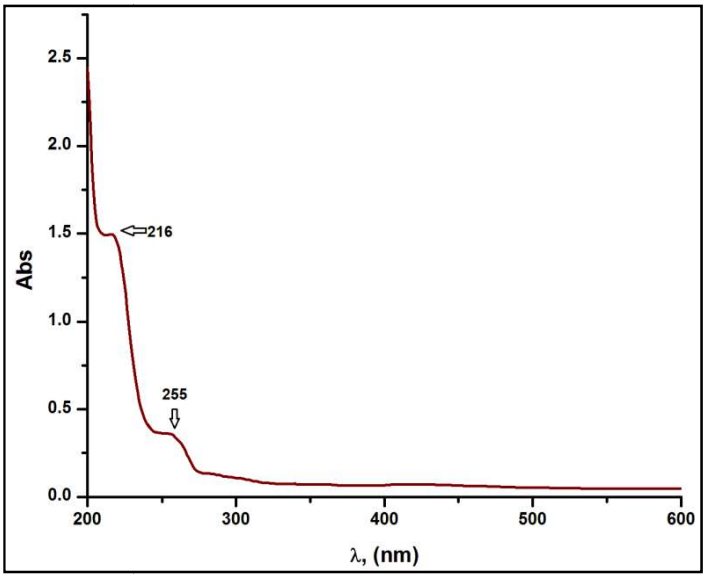


Fig 3.16. Absorption spectrum of Mono-6-deoxy-6-(toluene-3,4-diamino)-β-cyclodextrin (2).

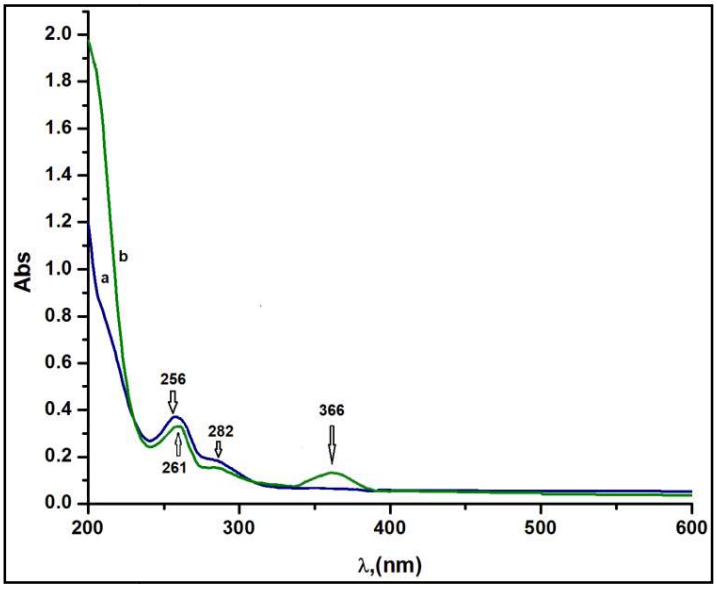


Fig 3.17. Absorption spectra of (a) Mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (3a) and (b) Zn complex of Mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (4a).

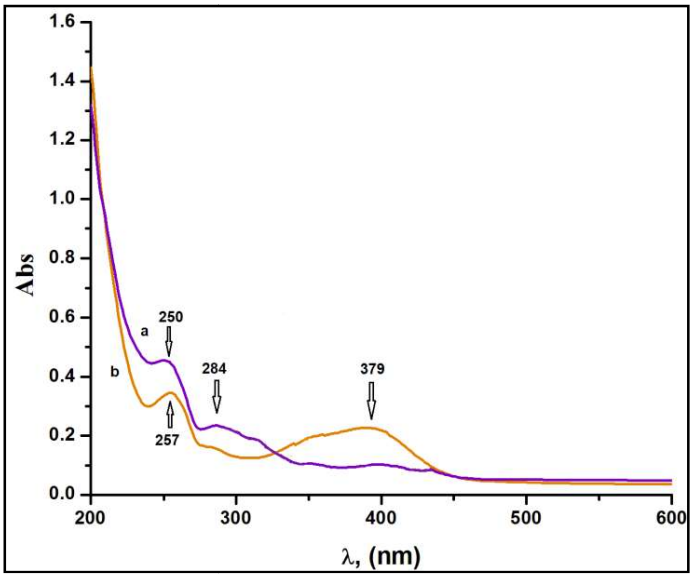


Fig 3.18. Absorption spectra of (a) Synthesis of Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (3b) and (b) Zn complex of Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (4b).

The peaks appearing at 366 nm for 4a and at 397 nm for 4b can be ascribed to charge transfer transition and the electronic spectra of the complexes suggested tetrahedral geometry for the complexes.

3.3.4. Stability constant and stochiometry of the complexes

The stability constant for the complexes (4a and 4b) were determined by UV-visible spectrophotometric titration following mole-ratio method. The changes in the absorption spectra of the ligands (3a and 3b respectively) (initially1×10^{-5} mol·L^{-1}) were recorded against concentrations of Zinc acetate (1×10^{-3} mol·L^{-1}) in aqueous solutions stepwise added by 2.5 μL. During the spectrophotometric titration, complex formation was indicated by mainly gradual increase in the intensity of 256 and 250 nm peaks for the ligands 3a and 3b, respectively as shown in (Figures 3.19 and 3.20). The analysis of the spectrophotometric data (using Mole-ratio plot) was performed with the absorbance values at λ = 256 nm & λ = 250 nm (for 3a & 3b, respectively) as shown in the insets of (Figures 3.19 and 3.20), which proves the stoichiometry of the complexes to be 1:1. Also using the ($c_M.c_L$/A *versus* c_M) data and following a literature method,[26] the stability constants $\log K_f$= 6.30 ± 0.02 & $\log K_f$= 6.33 ± 0.02 at 25 °C, were found for the complexes 4a and 4b, respectively.

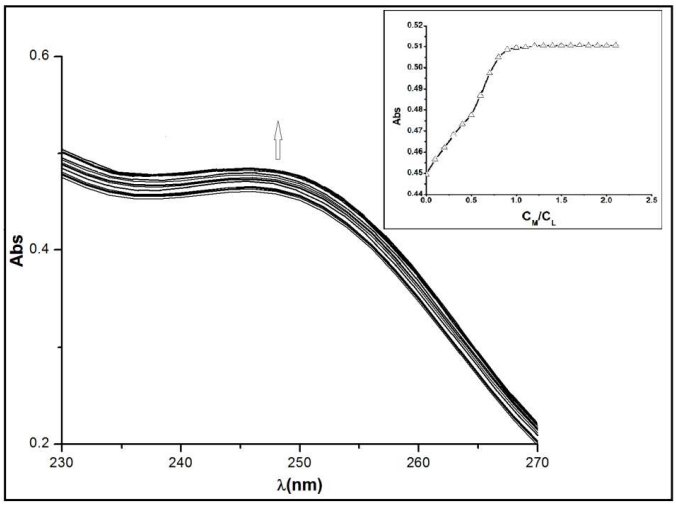


Fig 3.19. UV–Vis spectra of the ligand (**3a**) (1×10^{-5} mol·L^{-1}) in the presence of increasing concentrations of Zn(II) ions. Inset: Absorbance plot for the ligand (**3a**) with Zn(II) ion against *c*M:*c*L.

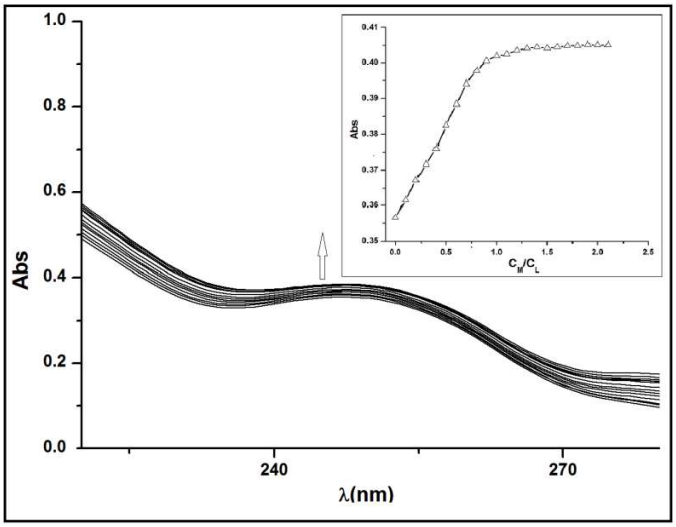


Fig 3.20. UV–Vis spectra of the ligand (**3b**) (1×10^{-5} mol·L^{-1}) in the presence of increasing concentrations of Zn(II) ions. Inset: Absorbance plot for the ligand (**3b**) with Zn(II) ion against *c*M:*c*L.

3.3.5. ESI-MS

The ESI-MS spectra of the ligands (3a and 3b) and complexes (4a and 4b) were recorded and shown in figure (Figures 3.21-3.24). The ligands 3a and 3b showed m/z peaks at 1377.32 and 1388.53, respectively, which corresponds to the $[M + H]^+$. In both Zn(II)-complexes (4a & 4b), the peak of at 1378.32 and 1388.53 were disappeared and a new peak appeared at 1519.35 & 1528.96 (m/z), respectively which represents molecular weight of complexes. Therefore these findings were in good agreement with the respective structures as already revealed by the elemental and other spectral analyses.

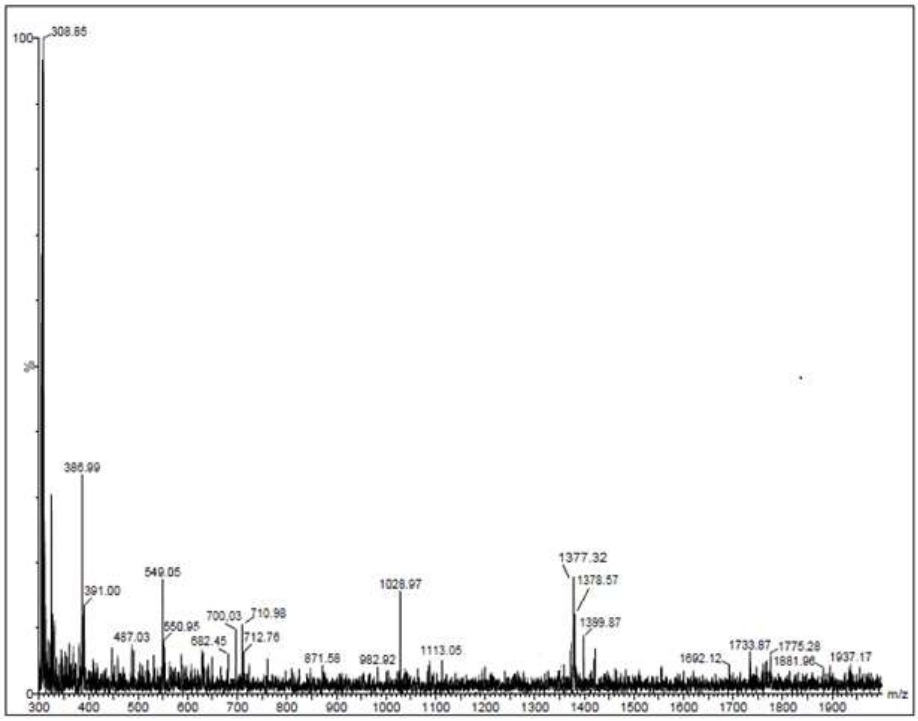


Fig 3.21. ESI-MS spectrum of Mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (3a).

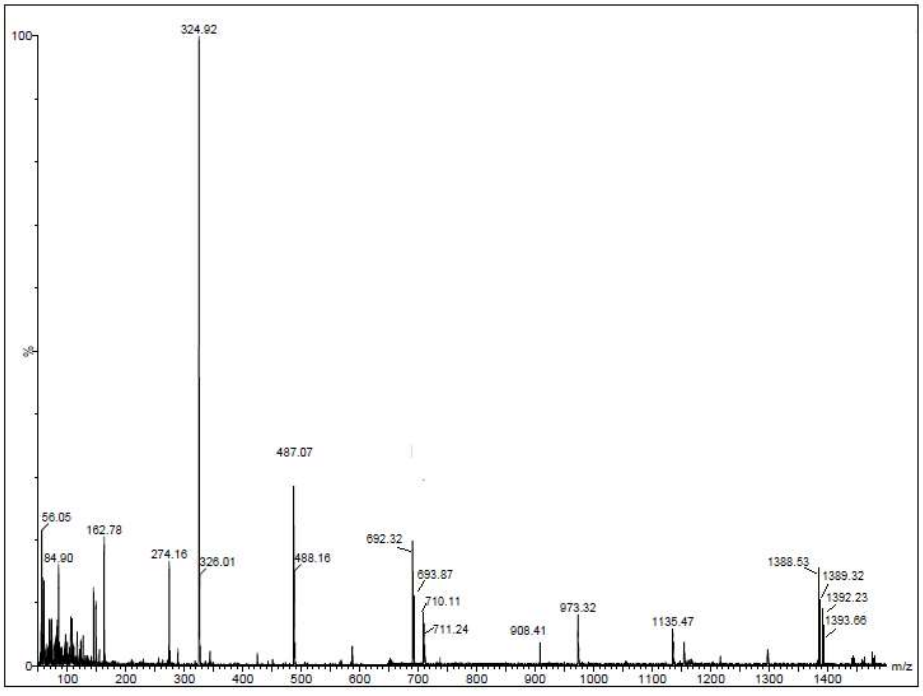


Fig 3.22. ESI-MS spectrum of Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (3b).

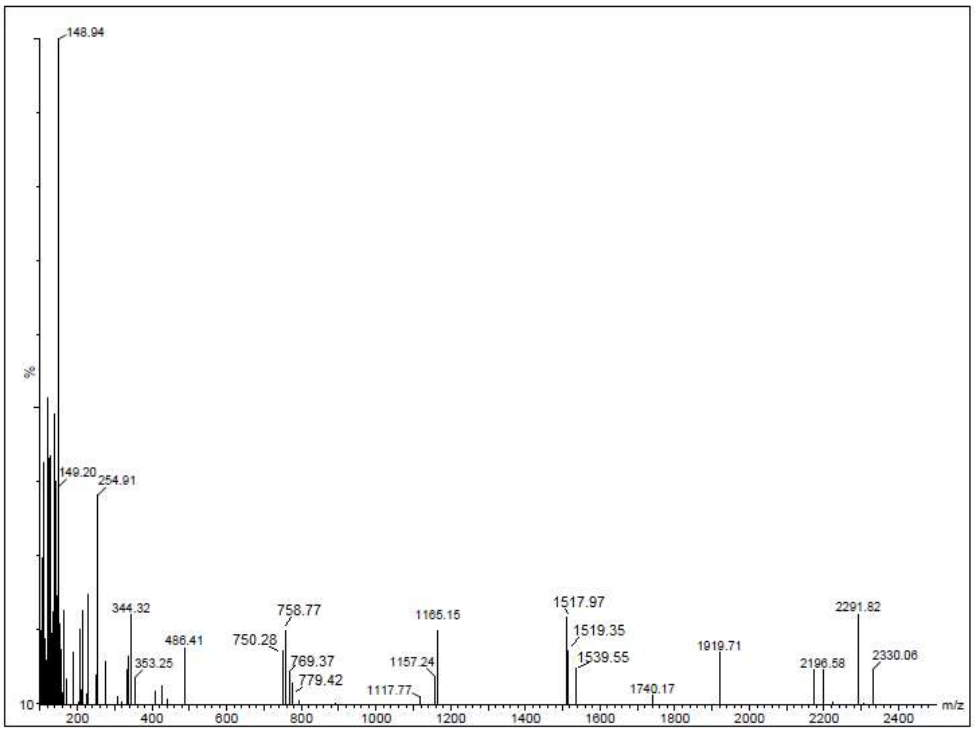


Fig 3.23. ESI-MS spectrum of Zn complex of Mono-6-deoxy-6-(4-(5-chloro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (4a).

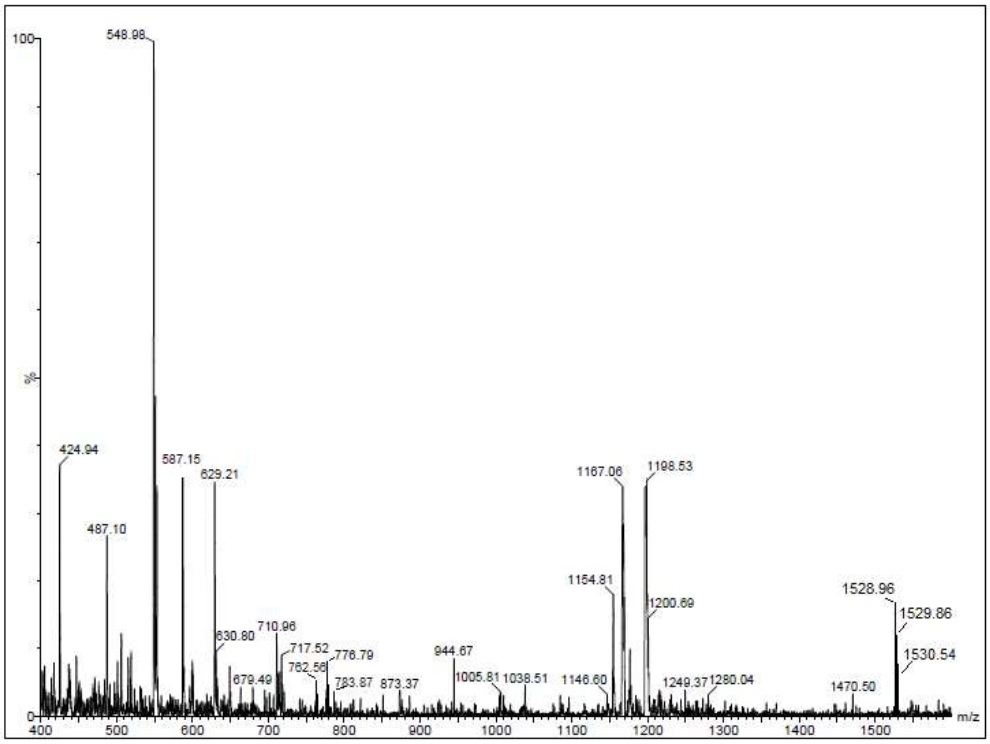


Fig 3.24. ESI-MS spectrum of Zn complex of Mono-6-deoxy-6-(4-(5-nitro-2-hydroxybenzylideneamino)-3,4-diaminotolune)-β-cyclodextrin (4b).

3.3.6. Antioxidant activity

There are several disorders directly or indirectly correlated to oxidative stress. Several external factors like, UV/X- rays, smoking, junk foods, mental stress, pollution, *etc*., can activate the chain reaction of ROS, these ROS can switch on many disorders and can activate the pathway of NF-kβ, responsible for triggering proinflammatory mediators. The mediators then cause the changes in Ca homeostasis and lead to hypertension. ROS can also affect many endocrine glands like pituitary and propagate several neuro-disorders like Alzheimer's disease, Perkinson's disease and so on. All the antioxidant activity measuring experiments were done with the both ligands (3a and 3b) and both complexes (4a and 4b) but ligands does not show appreciable results. So, here in this work only antioxidant activity of complexes (4a and 4b) has been discussed explicitly.

Table 3.1. IC_{50} values of β-cyclodextrin based Schiff base Zn(II) complex (4a and 4b) and standard for different antioxidant and free radical scavenging assays. Data expressed as mean ± S.D (n=6). $^{\alpha}$ $p<0.05$; $^{\beta}$ $p<0.01$; $^{\gamma}$ $p<0.001$; NS-Non significant when compared with respective standard.

Parameters	4a	4b	Standard
DPPH	$1273.37\pm57.18^{\gamma}$	$322.83\pm8.20^{\beta}$	203.20±1.97
Hydroxyl Radical	$116.68\pm0.67^{\gamma}$	$133.01\pm4.73^{\gamma}$	597.15±11.9
Hydrogen Peroxide	$146.87\pm6.21^{\beta}$	$283.67\pm4.81^{\beta}$	2185.22±187.45
Nitric Oxide	$857.71\pm139.92^{\alpha}$	$492.97\pm84.10^{\alpha}$	61.17±0.41
Superoxide Anion	$309.68\pm8.04^{\gamma}$	$249.53\pm34.30^{\alpha}$	94.59±3.75
Hypochlorous Acid	$691.38\pm53.95^{\beta}$	$262.60\pm2.37^{\gamma}$	130.07±5.16
Total Antioxidant Activity	$409.99\pm107.17^{\alpha}$	$603.45\pm36.95^{\beta}$	116.46±5.91
Lipid Peroxidation	$539.31\pm23.73^{\gamma}$	$513.20\pm3.55^{\gamma}$	11.16±0.26
Iron chelation	$343.09\pm64.96^{\alpha}$	$378.55\pm52.16^{\beta}$	1.49±0.02

In the present antioxidant profile, complex 4b exhibited higher free radical scavenging activity than the complex 4a as per DPPH assay (Figure 3.25.(A)). The percent of inhibition in case of complex 4b was (39.29 ± 0.41 %), where as 4a showed (13.85 ± 0.92 %) at the highest dose of 200 µg/mL concentration. This was evident from the discoloration of DPPH and low IC_{50} (Table 3.1) values in case of complex 4b (322.83

± 8.20). DPPH is a free radical that can accept an electron or hydrogen radical to become stable and reacts with reducing agent to form new bond, changing the color of the solution. The colored solution loses its color due to increased amount of antioxidant properties.[27] The elevated DPPH radical scavenging activity by complex 4b was due to the presence of significant antioxidant properties.

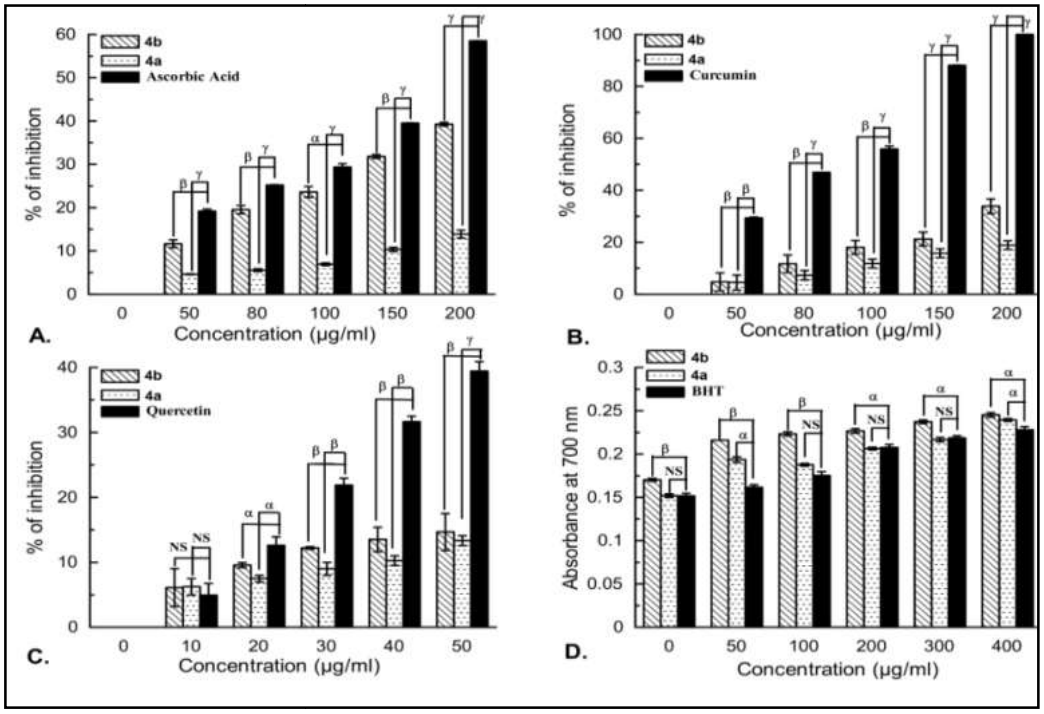


Fig 3.25. Antioxidant activity of β-cyclodextrin based Schiff base Zn(II) complexes (4a and 4b) (A) DPPH activity (B) Nitric oxide scavenging activity (C) Super oxide radical scavenging activity; (D) Reducing power assay. Data expressed as mean ± S.D (n = 6).
$^{\alpha}$ $p<0.05$; $^{\beta}$ $p<0.01$; $^{\gamma}$ $p<0.001$; NS-Non significant when compared with respective standard.

In nitric oxide scavenging activity (Figure 3.25.(B)), complex 4b showed moderate scavenging activities (33.88 ± 2.78 % at 200µg/ml) compared to the standard used (curcumin). Nitric oxide, produced from the amino acid L- arginine by the activation of nitric oxide synthase (NOS), is a potent mediator of pro-inflammatory cellular activation. Due to chronic inflammation, calcium dependent NOS produces excess amount of NO which can turn on tumor development. iNOS (calcium independent isoform of NOS) is activated by LPS (lipopolysachharide) and induced by the translocation of NF-κβ, leading to the progression of cancer.[28] Thus, complex (4b)

might inhibit the formation of inflammation related carcinogenesis by scavenging the nitric oxide.

The complex 4b was found to exhibit scavenging activity in case of superoxide anion (14.66 ± 2.83 at 200 µg/mL) (Figure 3.25(C)), hypochlorous acid (4b: 37.41 ± 0.60 % and 4a: 24.24 ± 0.57 % at 200 µg/mL) (Figure 3.26(D)) and lipid peroxidation (Figure 3.28(A)) scavenging assay in dose dependent manner and highly significant scavenging activity in case of hydroxyl radical (4b: 81.33 ± 2.36 % and 4a: 81.58 ± 2.23 % at 200 µg/mL) (Figure 3.26(A)) and hydrogen peroxide scavenging assay (4b: 42.03 ± 0.82 % and 4a: 75.15 ± 1.72 % at 200 µg/mL) (Figure 3.26.(B)). Hydroxyl radical, the short-lived free radical is most harmful and has the potential to damage our biological system.[27] Metal chelator may contribute to the reduction of $OH^{\cdot}$ by the oxidation of Fe^{2+} to Fe^{3+}. Another potential ROS, hydrogen peroxide (H_2O_2), is formed due to the mutation of superoxide anion or might be produced from superoxide ion in the presence of superoxide dismutase in the peroxisomes.[29-31]

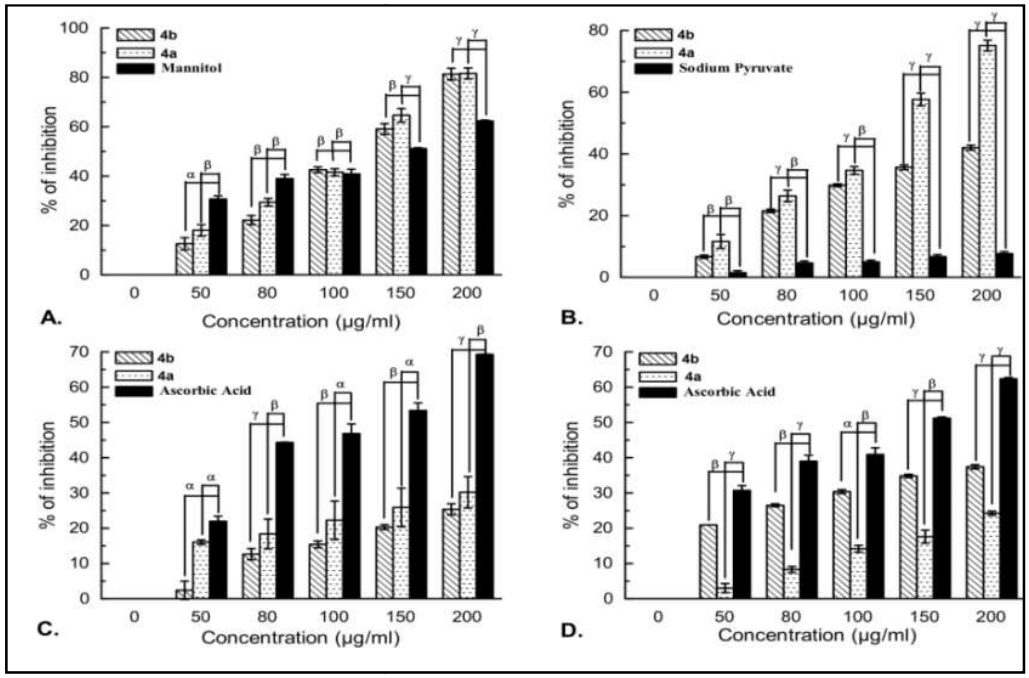


Fig 3.26. Antioxidant activity of β-cyclodextrin derived Schiff base Zn(II) complex (4a and 4b). (A) Hydroxyl radical scavenging assay (B) Hydrogen peroxide scavenging activity (C) Total antioxidant scavenging activity; (D) Hypochlorous acid scavenging assay. Data expressed as mean ± S.D (n=6). $^{\alpha}$ $p<0.05$; $^{\beta}$ $p<0.01$; $^{\gamma}$ $p<0.001$; NS- Non significant when compared with respective standard.

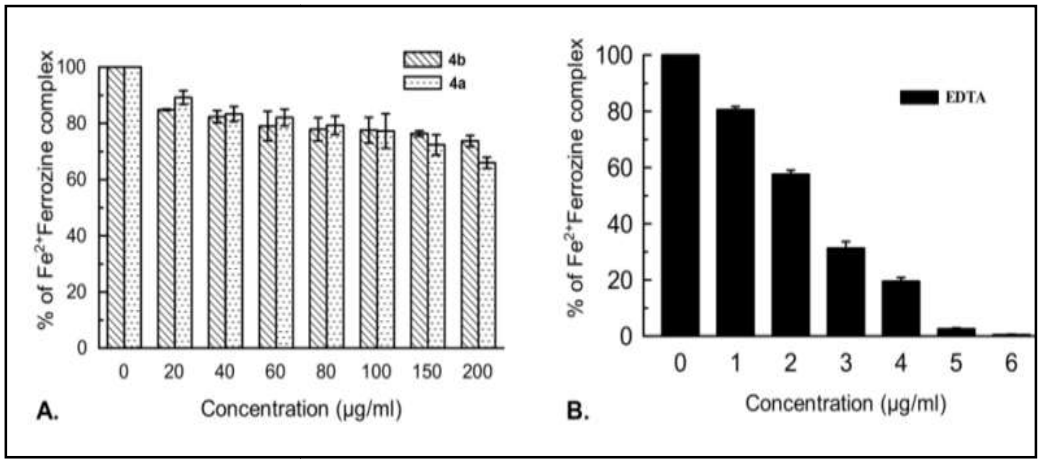


Fig 3.27. Antioxidant activity of β-cyclodextrin based Schiff base Zn(II) complex (4b and 4a). (A) Iron chelation by the complexes (B) Iron chelation by the standard (EDTA). Data expressed as mean ± S.D (n=6). $^{\alpha}$ $p<0.05$; $^{\beta}$ $p<0.01$; $^{\gamma}$ $p<0.001$; NS – Non significant when compared with standard.

Despite its lesser reactivity compared to other potential ROS, H_2O_2 performs an important role to modulate carcinogenesis. H_2O_2 defused throughout the mitochondria and cell membrane and subsequently generate various types of cellular injury.[29,30] In *in-vivo* condition hydroxyl radical ($OH^{\cdot}$) produces 8-hydroxy guanosine, the hydrolysis product of 8-hydroxydeoxyguanosine (8-OHdG), can attack on DNA and is involved in carcinogenesis progression, especially breast carcinoma.[32-34]Thus, increase in the scavenging activities of H_2O_2 and $OH^{\cdot}$ by the two synthesized complexes (4a & 4b) might facilitate chemoprevention. Spontaneous dismutation of the highly toxic superoxide anion ($O_2^{\cdot-}$) in mitochondria generates singlet oxygen. Generation of singlet oxygen from superoxide anion is one of the primary causes of lipid peroxidation. Lipid peroxidation is the pathogenesis of a wide range of diseases. Initiation of lipid peroxidation is the cause of propagation of chain reaction taking place until termination products are produced. Therefore, the end product malomdialdehyde (MDA), 4-hydroxy-2-nonenol and F2-isoprostanes were generated from the lipid peroxidation and accumulated in biological system. Accumulation of these toxic products can cause mutation and deletions in both nuclear and mitochondrial DNA. 4-hydroxy nonenal (4-HNE), the biomarkers of oxidative stress and are important in a number of cancer signaling pathways.[35] The present study demonstrated the significant superoxide anion and lipid peroxidation scavenging

capacities by the complexes, thus suggesting a probable protective role against carcinogenesis by the prevention of peroxidase formation. The neutrophilic enzyme,

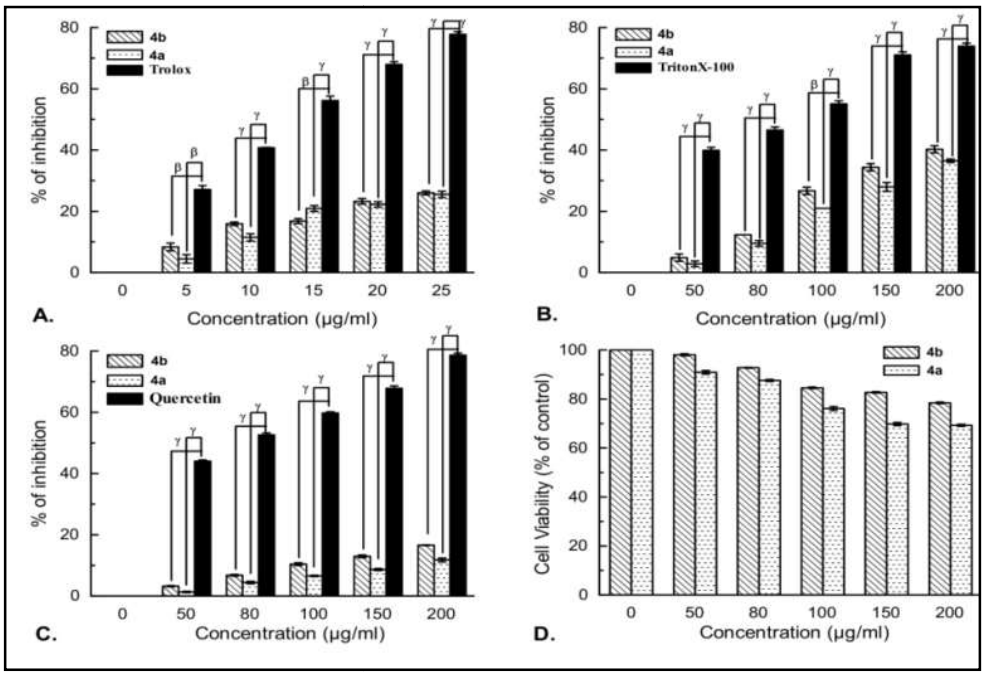


Fig 3.28. Antioxidant activity of β-cyclodextrin based Schiff base Zn(II) complex (4b and 4a). (A) Lipid peroxidation activity (B) Haemolytic assay (C) Erythrocyte membrane stabilizing activity (D) MTT cell proliferation assay. Data expressed as mean ± S.D (n=6). Data expressed as mean ± S.D (n=6). $^{\alpha}$ $p<0.05$; $^{\beta}$ $p<0.01$; $^{\gamma}$ $p<0.001$; NS – Non significant when compared with respective standard.

myeloperoxidase, resulting from the oxidation of Cl^- ions at the site of inflammation produces hypochlorous acid (HOCl) and cause target cell lyses.[36]The scavenging activity of hypochlorous acid by the two Zn(II) complexes (4a and 4b) appears quite promising and therefore, might be able to combat inflammation related carcinogenesis. Iron is a potential enhancer of ROS formation. In the present study complex 4b (73.69 ± 1.97 % at 200 µg/ml) and 4a (65.99 ± 2.01 % at200 µg/mL) were found to fade the color of ferrozine-complex, indicating its iron chelating capacity (Figure 3.27.(A)- Figure 3.27.(B)). Both the complexes 4b (0.24 ± 0.01 %) and 4a (0.23 ± 0.01 %) exhibited higher reducing power activities (Figure 3.25.(D)) than the standard BHT (0.22 ± 0.01 %). The total antioxidant activity (Figure 3.26.(C)) was significantly high in case of both the complex compared to the standard ascorbic acid. This result manifests crucial antioxidant scavenging activities by the

both Zn(II) complexes. Thus, the complex 4b might prove to be a key component in prevention of cancer in terms of oxidative stress and free radical generation.

Erythrocyte Membrane Stabilizing Activity (EMSA) (Figure 3.28.(C)) is another crucial assay which evaluates the antioxidant potentiality against the superoxide radical mediated damage of the erythrocyte membrane. Both the complexes displayed excellent membrane stabilizing activity, complex 4b (16.60 ± 0.09 % at 200 μg/mL) and complex 4a (11.76 ± 0.65 % at 200 μg/mL), by the inhibition of superoxide radical. Thus, membrane stabilizing activity by the complex 4b might aid to improve the immune system.[37] Hemolytic activity is one of the most crucial parameters of cytotoxicity. In the mentioned experiment complex 4b (40.25 ± 1.17 % at 200 μg/mL) and 4a (36.50 ± 0.54 % at 200 μg/mL) complex showed potential hemolytic activity (Figure 3.28.(B)). Besides, antioxidant capacity, both the complex were analyzed *in vitro* for immunomodulatory activity. MTT colorimetric assay was performed to determine the cytotoxicity of the synthesized complexes on mice spleenocytes (Figure 3.28.(D)). Spleen contains a relatively homogeneous fraction of B and T lymphocyte and thus, immune-proliferation of spleen provides an understanding of the influence of the synthesized complex on B and T cell lymphocyte. The B and T cell secrete several types of cytokines like IFN-γ, TNF-α, IL-4, IL-6, and IL-12 which are very essential for maintaining our immunological functions. β-cyclodextrin derived Schiff base Zn(II) complexes (4a & 4b) were observed negligible cytotoxic effect on spleenocytes. It can be concluded from the MTT assay the synthesized complex is not harmful for biological function as far as the present investigation is concerned. Thus, the synthesized complexes have the ability to modulate innate immune functions and have promising effects in alleviating oxidative stress related disorder.

3.4. Conclusion

In summary, incorporation of *β*-cyclodextrin moiety in the ligands structures of the complexes induced greater aqueous solubility and thus greater acceptability of the complexes through bio-fluids. The complexes with sufficient solubility in aqueous phase has shown negligible cytotoxic effects but moderate to good effects as anti-oxidant and they can thus help in combating oxidative stress related disorder leading to carcinogenic pathways, as per the result obtained from different biochemical analyses.

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