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SYNTHESIS, CHARACTERIZATION, DNA BINDING AND NUCLEASE ACTIVITY OF IRON(II) COMPLEXES OF ISONICOTINOYL HYDRAZONES

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ABSTRACT
A series of iron (II) complexes of isonicotinoyl hydrazones have been synthesized and characterized based on elemental analysis, molar conductivity, magnetic susceptibility measurements, infrared and electronic spectroscopy. Electrochemical behaviour of these complexes is investigated by cyclic voltammetry. The DNA binding constants K_b of the complexes are determined systematically with spectrophotometric titrations by using Calf Thymus DNA (CT-DNA). Cleavage activities of these complexes have been investigated on double stranded pBR322 plasmid DNA by gel electrophoresis in the absence and in presence of oxidant. The complexes behave as efficient chemical nucleases with hydrogen peroxide activation in the presence of reductant (DTT).

KEYWORDS: Bioinorganic Chemistry, iron (II) complexes, isonicotinoyl hydrazones, DNA binding, DNA cleavage.

INTRODUCTION

Transition metal ions play an important role in a number of chemical and biological reactions. The reactivity of metal complex can be modulated at high levels by simply changing the metal ions and their oxidation states. The successful synthesis and application of metal complexes can have a great impact on all areas of chemistry viz., organic, medicinal and biological chemistry.[1-3] Hydrazones and their metal complexes are found to have potential application in biology and medicine. Metal complexes of hydrazones are used as model compounds to mimic biological processes.[4-6]

Isonicotinoyl hydrazones play an important role in medicinal chemistry.[7] They are used as their complexes with metal ions for the treatment of number of diseases. As these organic compounds contain nitrogen as well as oxygen, they have the ability to form complexes easily with many metal ions. The hetero atoms, nitrogen as well as oxygen can form coordinate bonds with many metal ions and thus, form stable complexes. Number of reports is available on the medicinal importance of these compounds. Isonicotinoyl hydrazones have been used as anti-tuberculosis.[8, 9] and anticancer drugs.[10]

In the light of the above and in continuation of our ongoing research work, here in, we report synthesis, spectral characterization and DNA binding and cleavage activity of Transition metal complexes with a series of three INH ligands. Three ligands viz. 2-hydroxy benzaldehyde isonicotinoyl hydrazone (HBINH), 2,-hydroxy acetophenone isonicotinoylhydrazone (HAPINH) and 2,-hydroxy benzophenone isonicotinoyl hydrazone(HBPINH) are synthesized and characterized.

EXPERIMENTAL

MATERIALS AND METHODS

Isoniazid, 2-hydroxybenzaldehyde, 2-hydroxyacetophenone, 2-hydroxybenzophenone and agarose were purchased from Sigma-Aldrich. All other chemicals were of AR grade and used as provided. The solvents used for the synthesis were distilled before use. Calf –Thymus DNA (CT-DNA) was purchased from Genio Bio labs, Bangalore, India. Elemental analyses were carried out on a Heraeus Vario EL III Carlo Erba 1108 instrument. Magnetic measurements were taken at 298K using lakeshore VSM 7410 instrument. Molar conductivity measurements at 298 ± 2K in dry and purified DMF were carried out using a ELICO CM model 162 conductivity meter. The electronic spectra were recorded in DMF with a UV lamda50 (Perkin-Elmer) spectrophotometer. IR spectra were recorded in the range 4,000–400 cm^{-1} with a Perkin-Elmer spectrum100 spectrometer on KBr discs. Cyclic voltammetric measurements were taken on a CH instruments assembly equipped with an X–Y recorder. Measurements were taken on degassed (N_2 bubbling for 5 min) solutions (10^{-3} M) containing 0.1 M Bu_4NPF_6 as the supporting electrolyte. The three-electrode system

consisted of glassy carbon (working), platinum wire (auxiliary) and Ag/AgCl (reference) electrodes.

Preparation of Ligands

Ligands were prepared by reacting isoniazid with carbonyl compounds. A methanolic solutions of isonicotinylhydrazide (5mmol), carbonyl compound (5mmol) were mixed in a 100-ml round bottom flask. Two drops of HCl were added to the reaction mixture and refluxed for 3-6 hours. On cooling the reaction mixture to room temperature, yellow colored crystalline products were separated. The products were collected, washed with hot water and few drops of hexane and dried in vacuum. General structure of ligands is shown in Fig. 1.

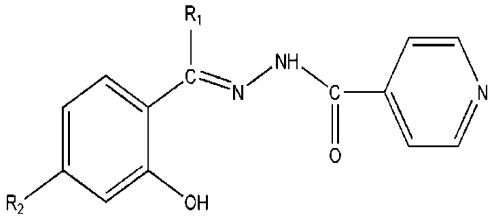

Fig 1: A general structure of ligands

]R_1	**R_2**	**Ligand**
H	H	HBINH
CH_3	H	HAPINH
C_6H_5	H	HBPINH

2-Hydroxy Benzaldehyde Isonicotinoyl hydrazone (HBINH): Yield 78%, M.Pt. 246-248 ^{0}C, Anal.(%) Calc. (found): C-64.73(64.42); H-4.56 (4.81); N-17.42 (17.64); ^{1}H-NMR spectra: δ(12.28) (singlet 1H), δ(11.07) (singlet 1H), δ(8.68) (singlet 1H), δ (7.5)(multiplet 4H) δ(6.5) (multiplet 4H), assigned to -OH, -NH, and =CH- , pyridine H, aromatic ring, protons respectively. Mass spectra of HBINH shows molecular ion peak at 241.

2-Hydroxy acetophenone isonicotinoyl hydrazone (HAPINH): Yield 72%, M.Pt.238-240 ^{0}C, Anal (%) Calc.(found): C-65.88(65.96); H-5.09 (5.02); N-16.47(16.64); ^{1}H-NMR spectra: δ(13.21) (singlet 1H), δ(11.59) (singlet 1H), δ(8.7)(multiplet 4H) δ (7.2) (multiplet 4H), δ(2.50) (singlet 3H), assigned to –OH, -NH, pyridine H aromatic ring, -CH_3 protons respectively. Mass spectra of HAPINH shows molecular ion peak at 255.

2-Hydroxy Benzophenone isonicotinoyl hydrazone (HBPINH): Yield 81%, M.Pt. 260-261^{0}C, Anal(%) Calc (found): C-71.92(71.86); H-4.73(4.60); N-13.24(13.45);. ^{1}H-NMR spectra: δ(10.36) (singlet 1H), δ(10.10) (singlet 1H),δ(8.7)(multiplet 4H) δ(7.2) (multiplet 5H), δ(6.8) (singlet 4H),assigned to –OH, -NH, pyridine H, aromatic ring protons respectively. Mass spectra of HBPINH shows molecular ion peak at 317.

Preparation of complexes

To a methanolic solution of ligand (5mmol), the aqueous solution of metal salt ($FeCl_2$. $4H_2O$) was added. The resulting solution was refluxed with stirring for one hour and then kept at room temperature and then filtered washed with methanol and dried in vacuo. The analytical data of all the complexes are given in Table 1. The ES$^+$I mass spectrum of Fe $(HBINH)_2$ complex are shown in Fig.2.

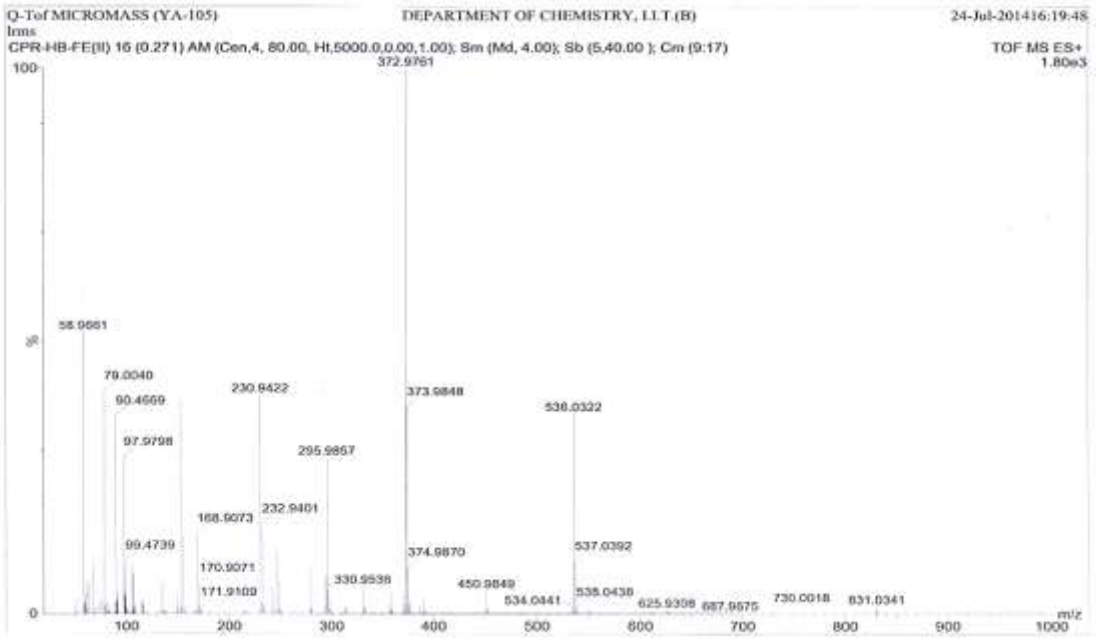

Fig 2: ES$^+$I Mass spectrum of [Fe $(HBINH)_2$].

DNA binding experiments

The interaction of the complexes with DNA was studied in tris-buffer medium. Solution of calf thymus DNA (CT-DNA) in (50mM NaCl/5 mM Tris-HCl; pH =7.0) buffer medium gave absorbance ratio at 260 nm and 280 nm of 1.85, indicating that the DNA was sufficiently free of proteins.[11] The DNA concentration per nucleotide was determined by absorption coefficient (6600 dm^3 mol^{-1} cm^{-1}) at 260 nm.[12] Stock solutions stored at 4ºC were used after no more than four days. The electronic spectra of metal complexes were monitored in the absence and presence of CT-DNA. Absorption titrations were performed by maintaining the metal complex concentration $2x10^{-5}$M and varying nucleic acid concentration. Absorption spectra were recorded after each successive addition of DNA solution. The intrinsic binding constant (K_b) was calculated by the equation, $[DNA]/\varepsilon_a-\varepsilon_f = [DNA]/\varepsilon_a-\varepsilon_f + 1/K_b (\varepsilon_a-\varepsilon_f)$, where [DNA] is the molar concentration of DNA in base pairs , ε_a, ε_b, ε_f are apparent extinction coefficient($A_{obs}/[M]$), the extinction coefficient for the metal (M) complex in the fully bound form and the extinction coefficient for free metal (M) respectively. A plot of [DNA] / $(\varepsilon_a-\varepsilon_f)$ versus [DNA] gave a slope of $1/(\varepsilon_a-\varepsilon_f)x$ K_b is the ratio of the intercept.

DNA cleavage studies

Cleavage experiments of supercoiled pBR322 DNA (300mg, 50µM) were carried out in presence of complex ($5X10^{-6}$M) separately in buffer solution (50mMTris-Hcl/NaCl), at P^H 7.2, followed by agarose gel electrophoresis. The samples were incubated for 30 min at 37°C. A loading buffer solution containing 25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol was added and electrophoresis was carried out in Tris-HCl buffer using 0.8% agarose gel containing 100µg/mL ethidium bromide. The reaction was monitored in the presence of activators Hydrogen peroxide (H_2O_2), and Dithiothreitol (DTT). The inhibition reactions were carried out by adding the reagent prior to the addition of the complexes. The standard protocols were followed for these experiments. The samples were incubated for 30 min at 37°C. Electrophoresis was performed at 75V in TBE buffer until the bromophenol blue reached to3/4 of the gel and gels were visualized by photographing the fluorescent ethidium bromide under a UV illuminator. The cleavage efficiency was measured by the ability of complex to convert supercoiled DNA (SC or Form I) to nicked circular form (NC or Form II) and linear form (LC or Form III).

RESULTS AND DISCUSSION

Elemental analysis, molar conductivity measurements and magnetic moment

Physical properties of complexes are given in **Table 1.** All the complexes are stable at room temperature, non-hygroscopic, slightly soluble in water, but more soluble in methanol, ethanol and readily soluble in CH_3CN, DMF and DMSO. The analytical data are consistent with the proposed molecular formulae of complexes. Low molar conductivity values of present complexes suggest non-electrolytic nature of the complexes. The effective magnetic moments (μ_{eff}) of the iron(II) complexes (1-6) lie in the range 5.15-5.24 B.M. at room temperature. The values suggest high spin octahedral geometry for the complexes.[13]

Table 1: Physico-chemical properties of iron(II) complexes

Complex	Melting Point ºC	Elemental analysis found(cal.)%			μ_{eff}	Λ_M^a
		Carbon	Hydrogen	Nitrogen		
$Fe(HBINH)_2$	295-296	57.54(58.20)	3.25(3.73)	15.01(15.67)	5.21	4.3
$Fe(HAPINH)_2$	268-270	58.31(59.37)	4.46(4.25)	14.27(14.89)	5.15	15.9
$Fe(HBPINH)_2$	>300	67.31(66.27)	4.18(4.06)	12.89(12.20)	5.24	13.4

Electronic spectra

Electronic absorption spectra of iron(II) complexes were recorded in DMF. The important electronic spectral data of iron(II) complexes are presented in **Table 2**. The electronic spectrum of $Fe(HBPINH)_2$ complex is shown in **Fig 3.** All the complexes show strong intense bands in the region 34129-35103 cm^{-1} attributed due to intraligand and π-π* aromatic ring. Another sharp peak shows at the region of 27394-28632 cm^{-1} is due to n-π* transition. One medium intensity band observed in the range 17857-21739 cm^{-1} is due to metal to ligand charge transfer transition (MLCT). A weak band is observed in the region of 10504-11534 cm^{-1} is due to d-d transition which is assigned to the $^5T_{2g} \rightarrow {}^5E_g$ transition in octahedral field.

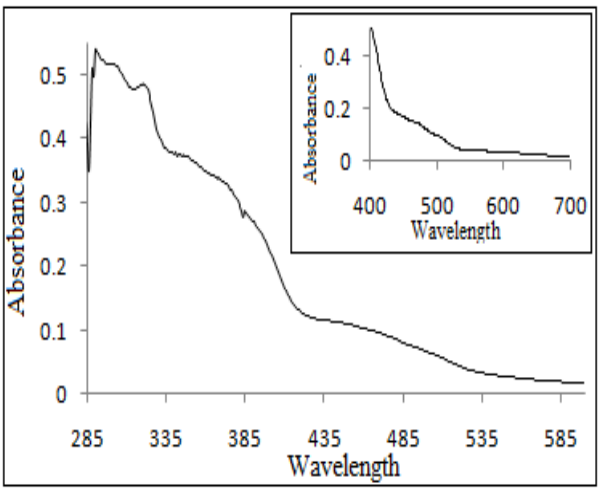

Fig. 3: Electronic spectrum of $[Fe(HBINH)_2]$

Table 2: Electronic Spectral data(cm^{-1}) of Iron(II) complexes

Complex	π-π^* tansition	n-π^* ransition	CT transition	d-d transition
$Fe(HBINH)_2$	34255	27394	18409	11534
$Fe(HAPINH)_2$	35103	28632	17857	10504
$Fe(HBPINH)_2$	34129	27700	21739	10915

IR spectra

The important bands in infrared spectra of the ligands and their metal complexes are discussed. Important IR spectral bands of complexes are presented in **Table 3**. An intense band is observed in the region of 3180-3398 cm^{-1} in IR spectra of ligands due to phenolic ν(-OH) group. The band is disappeared in iron complexes. This indicates deprotonation of phenolic group and band formation between phenolic oxygen and iron ion.[14-16] In ligands a strong band is observed in the region of 1698-1645 cm^{-1} which is assigned to ν(C=O)group. In the spectra of iron complexes this peak is shifted (8-25cm^{-1}) to lower wave numbers suggesting the involvement of >C=O group in chelation. The C=N (imine) vibration is observed in 1509-1631 cm^{-1} range in the IR spectra of ligands. This band is shifted to lower wave number in IR spectra of all the complexes suggesting the participation of azomethine nitrogen atom in coordination with iron ion. The non-ligand absorption bands occurring in the regions 561-508 cm^{-1} and 403-428 cm^{-1} are assigned to $\nu_{(M-O)}$ and $\nu_{(M-N)}$ vibrations respectively.[17] General structure of iron(II) complexes are shown in **Fig 4.**

Table 3: IR spectral data of Iron(II) complexes

Compound	ν(O-H) cm^{-1}	ν(NH) cm^{-1}	ν(C=O) cm^{-1}	ν(C=N) cm^{-1}	ν(C-O) cm^{-1}	ν (M-O) cm^{-1}	ν (M-N) cm^{-1}
HBINH	3180	3003	1698	1613	1274	-	-
$Fe(HBINH)_2$	-	3064	1673	1555	1248	561	408
HAPINH	3234	3015	1645	1609	-	-	-
$Fe(HAPINH)_2$	-	3002	1636	1573	1219	542	410
HBPINH	3398	3104	1669	1615	-	-	-
$Fe(HBPINH)_2$	-	3015	1648	1602	1243	519	428

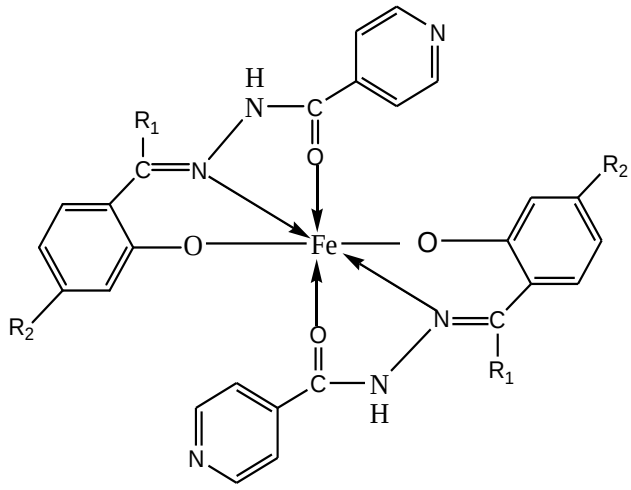

Fig. 4: Structure of metal complexes.

Cyclic voltammetry

The redox behavior of the complexes has been investigated by cyclic voltammetry in DMF using 0.1 M tetrabutylammonium hexafluorophosphate as supporting electrolyte. The cyclic voltammogram of $Fe(DAPINH)_2$ complex is given in **Fig 5.** The electrochemical data of iron(II) complexes are presented in **Table 4.** The data reveal that Iron(II) complexes have single cathodic wave, corresponding to one electron reduction Fe(II)→Fe(I). The reduction is reversible which occurs in the range -0.956 to -0.698 vs Ag/AgCl reference electrode. The separation between cathodic and anodic peaks (ΔE = 115-272 mV) indicates quasi-reversible character. The potential difference $\Delta Ep = Ep_c - Ep_a$ in all the complexes exceeds the Nerstian requirement of 59/n mV (n = number of electrons involved in oxidation-reduction) which further suggests quasi-reversible character of the electron transfer reaction.

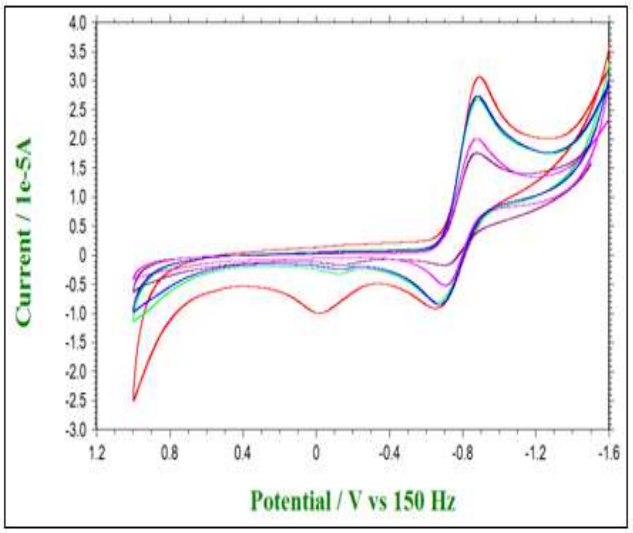

Fig 4: Cyclic voltammograms of $[Fe(HBPINH)_2]$ at different scan rates 25,50,75,100mVs^{-1}

Table 4: Cyclic voltammetric data of iron(II) complexes.

Complex	Redox couple	$E_{pc}V$	$E_{pa}V$	ΔE (mV)	$E_{1/2}$	$logK_c^a$	$-\Delta G^0$
$Fe(HBINH)_2$	II/I	-0.956	-0.841	115	-0.898	0.292	1677
$e(HAPINH)_2$	II/I	-0.892	-0.620	272	-0.756	0.123	709
$Fe(HBPINH)_2$	II/I	-0.863	-0.704	159	-0.783	0.211	1213

DNA binding studies

The binding interactions of the complexes with DNA were monitored by comparing their absorption spectra with without CT-DNA. Typical absorption spectra of complex $Fe(HAINH)_2$ in the absence and in the presence of CT DNA are shown in **Fig 6.** It has been observed that for each addition of CT-DNA to all the complexes shows a decrease in molar absorptivity (hypochromism ,Δε, +13.56 to +41.57%, **Table 5**) of the π-π^* absorption band as well as a bathochromic shift of a few nm(1-5nm). The intrinsic binding constant(K_b), was determined by using the equation. The intrinsic binding constants of iron complexes are given in **Table 5.**

$$\frac{[DNA]}{\varepsilon_a - \varepsilon_f} = \frac{[DNA]}{\varepsilon_a - \varepsilon_f} + \frac{1}{K_b}(\varepsilon_a - \varepsilon_f) \quad \text{----------(1)}$$

Hypochromism results from the contraction of DNA in the helix axis as well as from the change in conformation on DNA. Spectral changes suggest due to intercalative mode of binding of iron complex involving strong stacking interactions of complex between nitrogenous base pairs of DNA.[18]

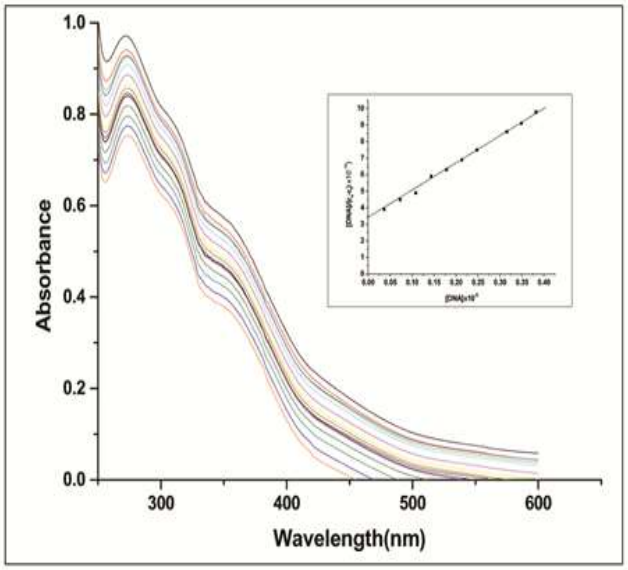

Fig 6: Absorption spectra of $[Fe(HAPINH)_2]$ in the absence and in the presence of increasing concentration of CT-DNA; top most spectrum is recorded in the absence of DNA and below spectra on addition of 10 µl DNA each time; A plot of [DNA]/ (ε_a- ε_f) vs. [DNA] is shown in the inset.

Table 5: Electronic absorption data upon addition of CT-DNA to the complexes

Complex	λ_{max} (nm)		Δλ/ nm	H%	K_b (M^{-1})
	Free	Bound			
Fe $(HBINH)_2$	354	357	3	+27.99	$3.59x10^5$
Fe $(HAPINH)_2$	272	273	1	+13.56	$6.20x10^6$
Fe $(HBPINH)_2$	375	378	3	+21.45	$3.8x10^6$

DNA cleavage studies

Nuclease activity of iron(II) complexes has been studied by agarose gel electrophoresis using pBR 322 plasmid DNA in Tris–HCl/NaCl(50 mM/5 mM) buffer (pH-7) in the presence and absence of H_2O_2 and DTT after 30 min incubation period at 37°C. **Fig 7** show the cleavage activity of iron (II) complexes. Iron complexes of shows high nuclease activity {except $Fe(HBINH)_2$}. In the presence of H_2O_2, the complexes cleave supercoiled DNA(Form I) into linear DNA (Form II) and nicked (Form III) [Figures 7]. Cleavage activity increases in the presence of H_2O_2 to metal complex. It is due to the reaction of iron complex ion with H_2O_2 thereby producing diffusible hydroxyl radicals which are capable of damaging DNA by two well known pathways: (1) the Fenton and the (2)Haber–Weiss mechanisms.[19,20] The necessity for a reductant for the cleavage of DNA by iron complexes indicates that Fe (II) ions are being reduced to Fe(I) ions, which are susceptible to oxidation.[21,22]

These OH^- free radicals participate in the oxidation of the deoxyribose moiety.[23] The mechanistic pathway for the above reaction as follows.

$Fe^{2+} + H_2O_2 \rightarrow Fe^{+} + HOO\bullet + H^{+}$ ……(1)

$Fe^{+} + H_2O_2 \rightarrow Fe^{2+} + OH^- + \bullet OH$ ……(2)

On comparision of lanes 4,7 and 10 with lanes 5,8 and 11, it is clear that the cleavage rate is enhanced in the presence reductant(DTT). The Fe(II) formed in the second step is reduced to Fe(I) by DTT, and reduced Fe(I) ion react with H_2O_2 and produces more hydroxyl radicals.[24] The oxidative cleavage becomes catalytic in the presence of reductant as indicated below. This observation provides an evidence for the oxidative cleavage of DNA.

$2\ Fe^{2+} + R(SH)_2 \rightarrow 2\ Fe^{+} + R(SS) + 2H^{+}$

$2Fe^{+} + H_2O_2 \rightarrow Fe^{2+} + OH^- + \bullet OH$

Where $R(SH)_2$ is DTT(Dithiothreitol)

Finally we conclude that nuclease activity of complexes become catalytic in the presence of H_2O_2 & DTT reagents.

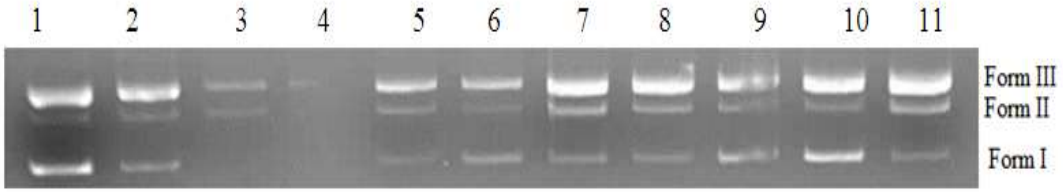

Fig. 7 Agarose gel (0.8%) showing results of electrophoresis of 1 µl of pBR 322 Plasmid DNA; 4 µl of Tris–HCl/NaCl (50 mM/5 mM) buffer (pH-7); 2 µl of complex in DMF($1x10^{-3}$ M); 11 µl of sterilized water; 2 µl of H_2O_2 (total volume 20 µl) were added, respectively, incubated at 37°C (30 min);

Lane 1:DNAcontrol; Lane 2: DNA control + H_2O_2; Lane 3: $[Fe(HBINH)_2]$ + DNA; Lane 4: $[Fe(HBINH)_2]$ + DNA + H_2O_2; Lane 5: $[Fe(HBINH)_2]$ + DNA +H_2O_2+ DTT; Lane 6: : $[Fe(HAPINH)_2]$ + DNA; Lane 7: $[Fe(HAPINH)_2]$ + DNA+H_2O_2; Lane 8: $[Fe(HAPINH)_2]$ + DNA+H_2O_2+DTT; Lane 9: $[Fe(HBPINH)_2]$+ H_2O_2 + DNA ; Lane 10: $[Fe(HBPINH)_2]$ + DNA + H_2O_2; Lane 11: $[Fe(HBPINH)_2]$ + DNA + H_2O_2+ DTT

CONCLUSIONS

Iron(II) complexes of a new series of isonicotinoylhydrazones bearing phenolic group have been synthesized and characterized based on physico-chemical and spectral studies. Physico-chemical data revealed that the complexes have general formula FeL_2 (where L = hydrazone). The hydrazones act as uni-negative tridentate ligand. Electronic spectral data suggest that the complexes have octahedral geometry. Absorption titrations suggest that the complexes bind DNA through intercalation involving a strong π -stacking interaction of the aromatic chromophore of complex between base pairs of DNA. In presence of H_2O_2, the complexes cleave DNA effectively. It may be due to the reaction of hydroxyl radical with DNA. In the presence of DTT and H_2O_2, complexes cleave DNA effectively suggesting that the complexes cleave DNA by oxidative path.

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