Supporting Information

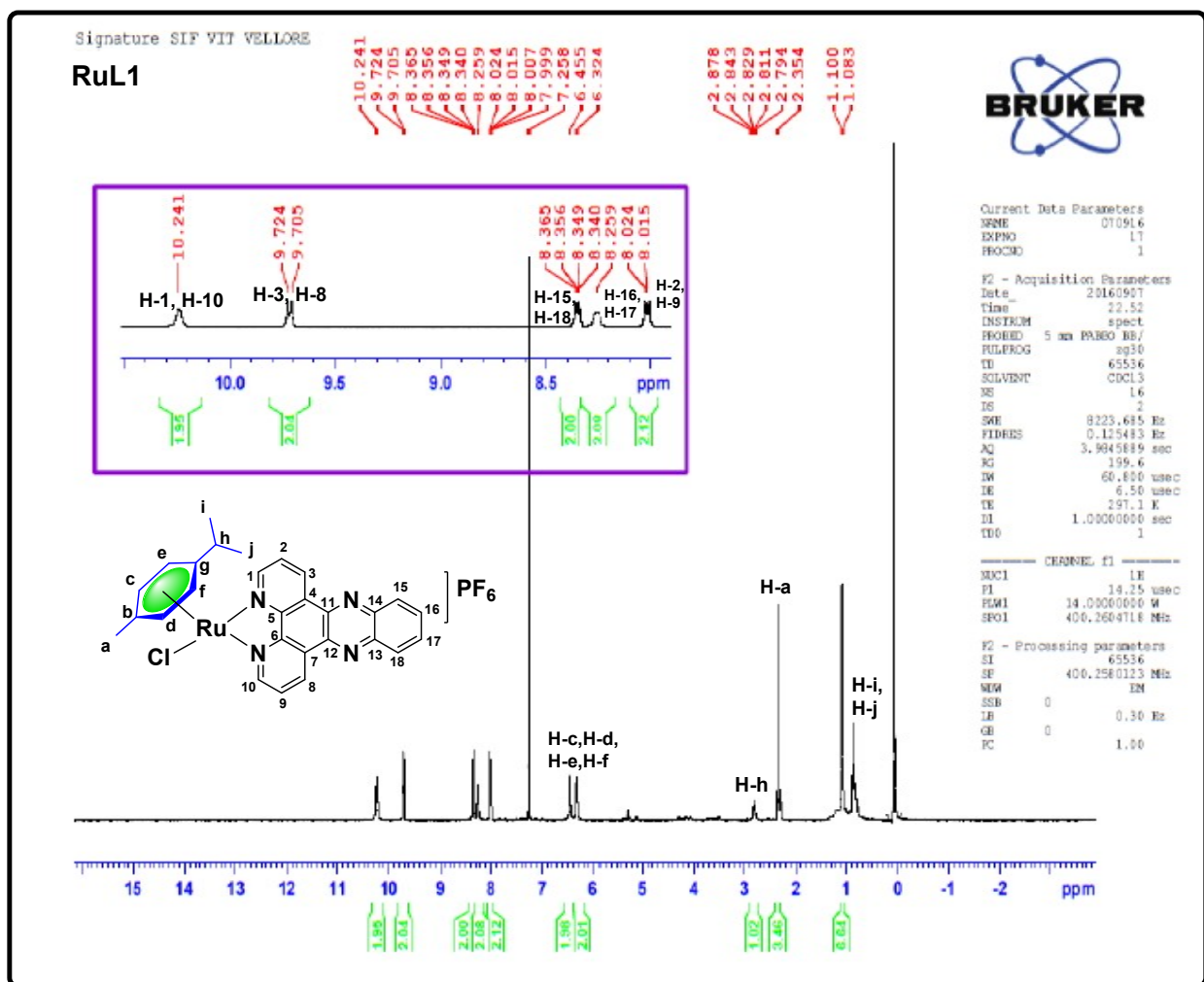## One pot three component synthesis of DNA targeting phototoxic Ru(II)-*p*-cymene dipyrido[3,2-a:2',3'-c]phenazine analogues
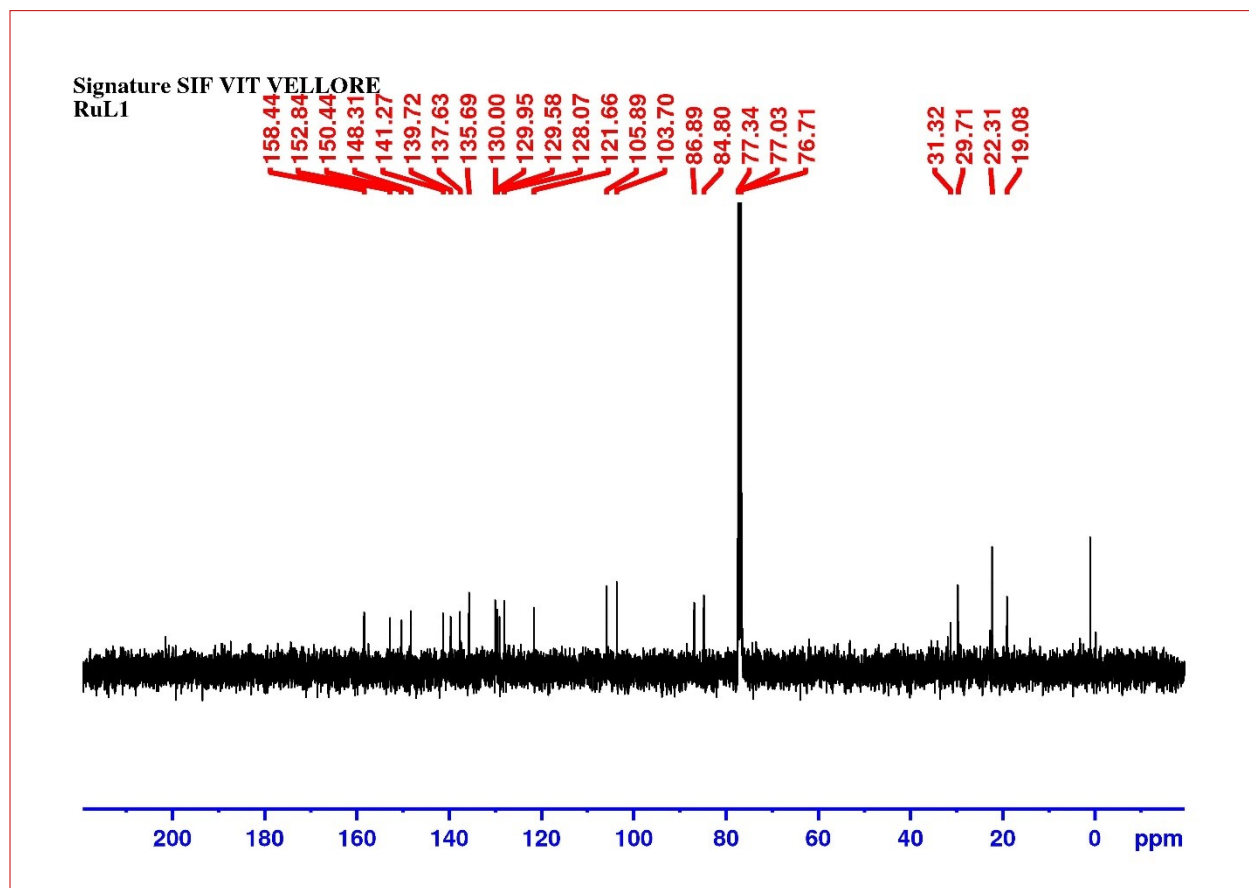
Binoy Kar, Priyankar Paira*

CHARACTERIZATION OF COMPLEXES (RuL1-RuL5):

^{1}H NMR of complex RuL1:

¹³C NMR of complex RuL1:

Signature SIF VIT VELLORE
RuL1

Peaks: 158.44, 152.84, 150.44, 148.31, 141.27, 139.72, 137.63, 135.69, 130.00, 129.95, 129.58, 128.07, 121.66, 105.89, 103.70, 86.89, 84.80, 77.34, 77.03, 76.71, 31.32, 29.71, 22.31, 19.08

^{19}F NMR of complex RuL1:

Signature SIF VIT VELLORE
RuL1

-69.18
-71.07

0	-20	-40	-60	-80	-100	-120	-140	-160	-180	-200	ppm

³¹P NMR of complex RuL1:

Signature SIF VIT VELLORE
RuL1

-135.43
-139.82
-144.21
-148.60
-152.99
-157.38

100   50   0   -50   -100   -150   -200   ppm

5

HRMS spectrum of complex RuL1:

24062022_BR_1 148 (2.599) AM2 (Ar,22000.0,556.28,0.00,LS 3); Cm (145:152)
1: TOF MS US+
2.84e8

Calculated mass m/z = 553.0733

Observed mass m/z = 553.0750

24062022_BR_1 148 (2.599) AM2 (Ar,22000.0,556.28,0.00,LS 3); Cm (145:152)
1: TOF MS US+
2.84e8

^{1}H NMR of complex RuL2:

^{13}C NMR of complex RuL2:
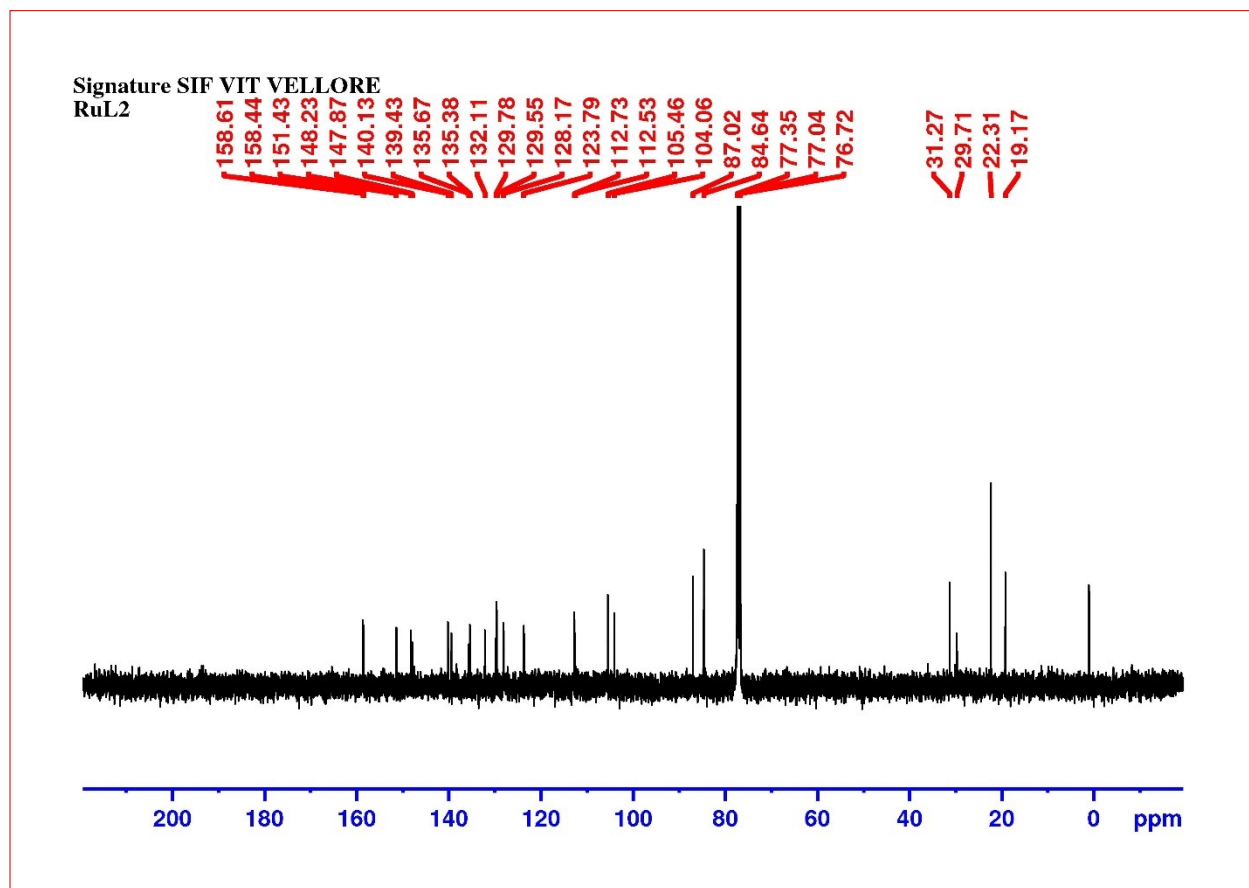
Signature SIF VIT VELLORE
RuL2

158.61, 158.44, 151.43, 148.23, 147.87, 140.13, 139.43, 135.67, 135.38, 132.11, 129.78, 129.55, 128.17, 123.79, 112.73, 112.53, 105.46, 104.06, 87.02, 84.64, 77.35, 77.04, 76.72, 31.27, 29.71, 22.31, 19.17

200   180   160   140   120   100   80   60   40   20   0   ppm

^{19}F NMR of complex RuL2:

Signature SIF VIT VELLORE
RuL2

−61.41
−69.19
−71.07

0 -20 -40 -60 -80 -100 -120 -140 -160 -180 ppm

³¹P NMR of complex RuL2:

Signature SIF VIT VELLORE
RuL2

-135.43
-139.82
-144.21
-148.60
-152.99

HRMS spectrum of complex RuL2:

Calculated mass m/z = 571.0639

Observed mass m/z = 571.0657
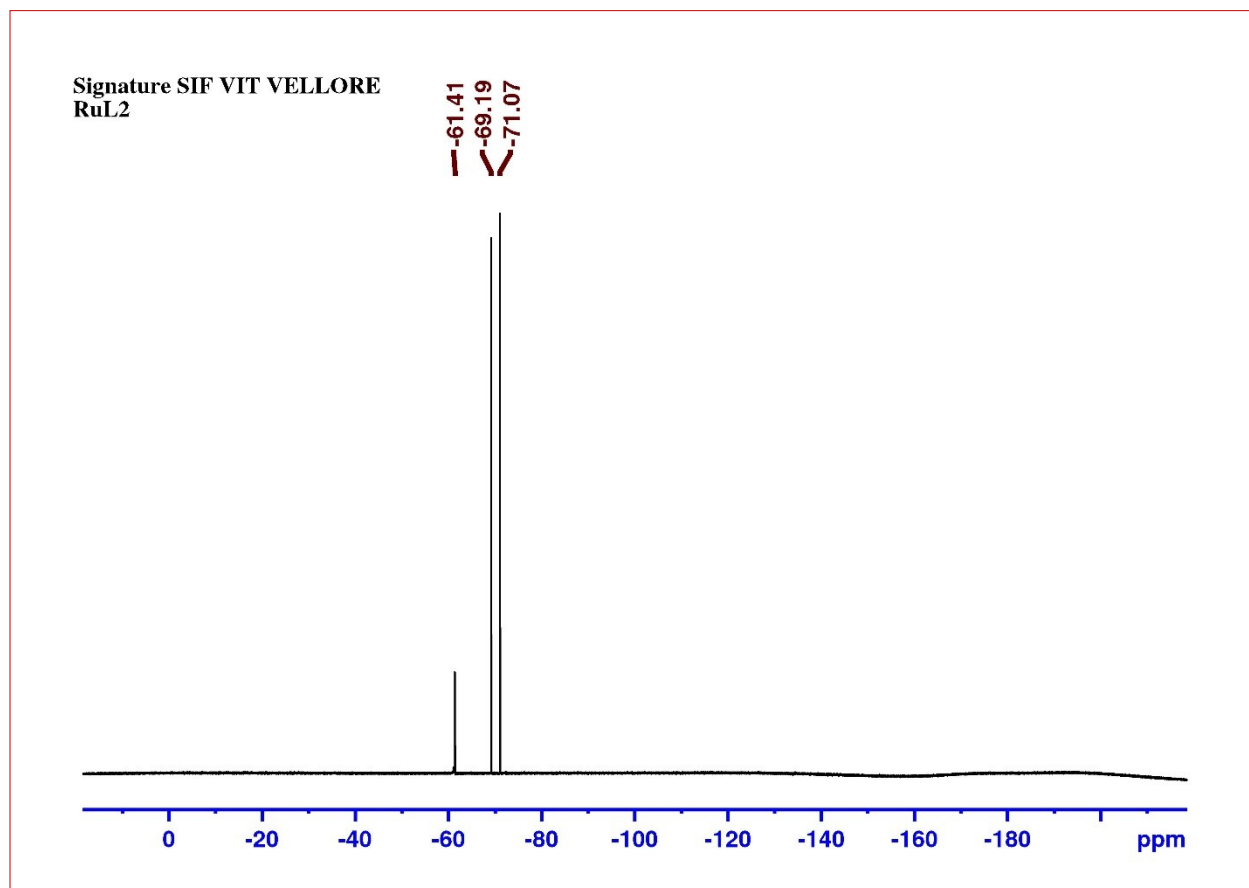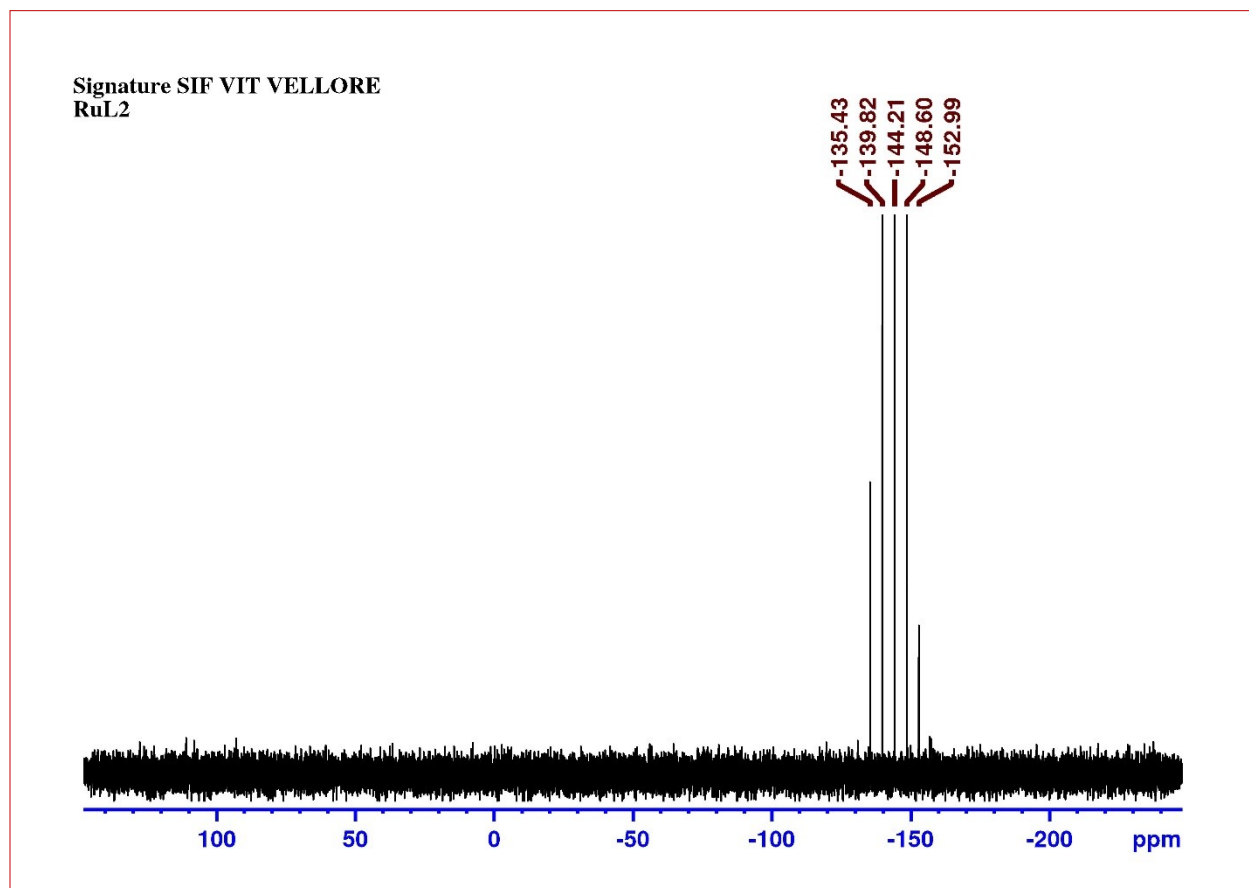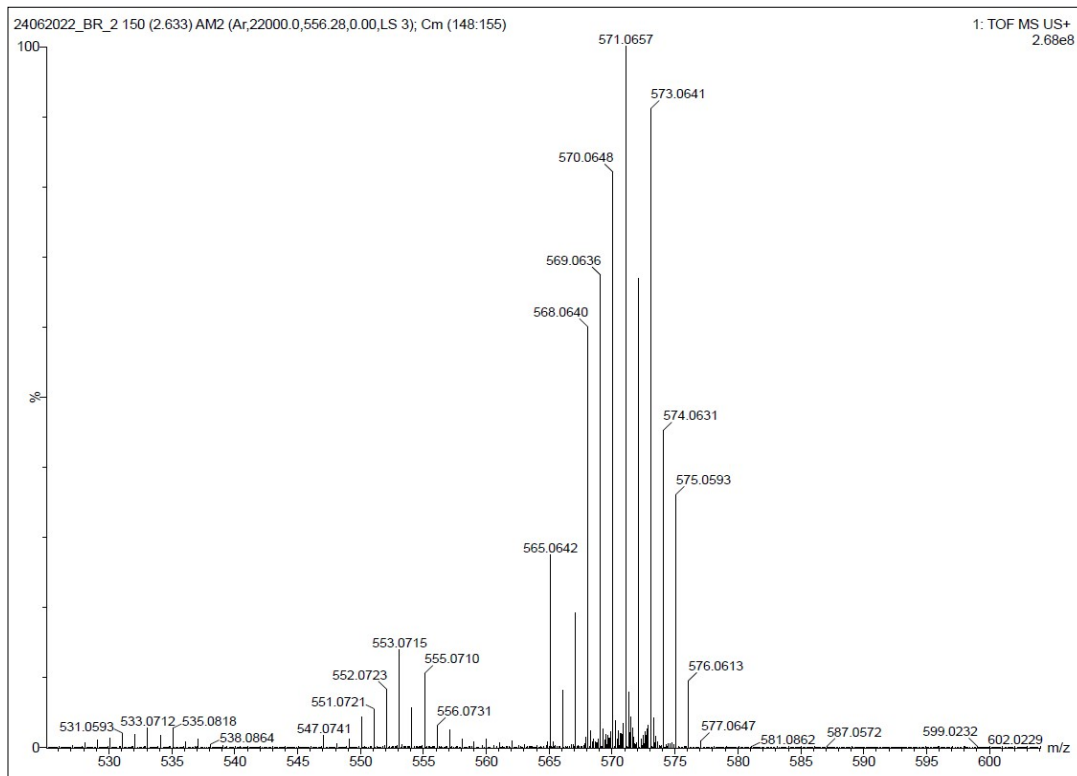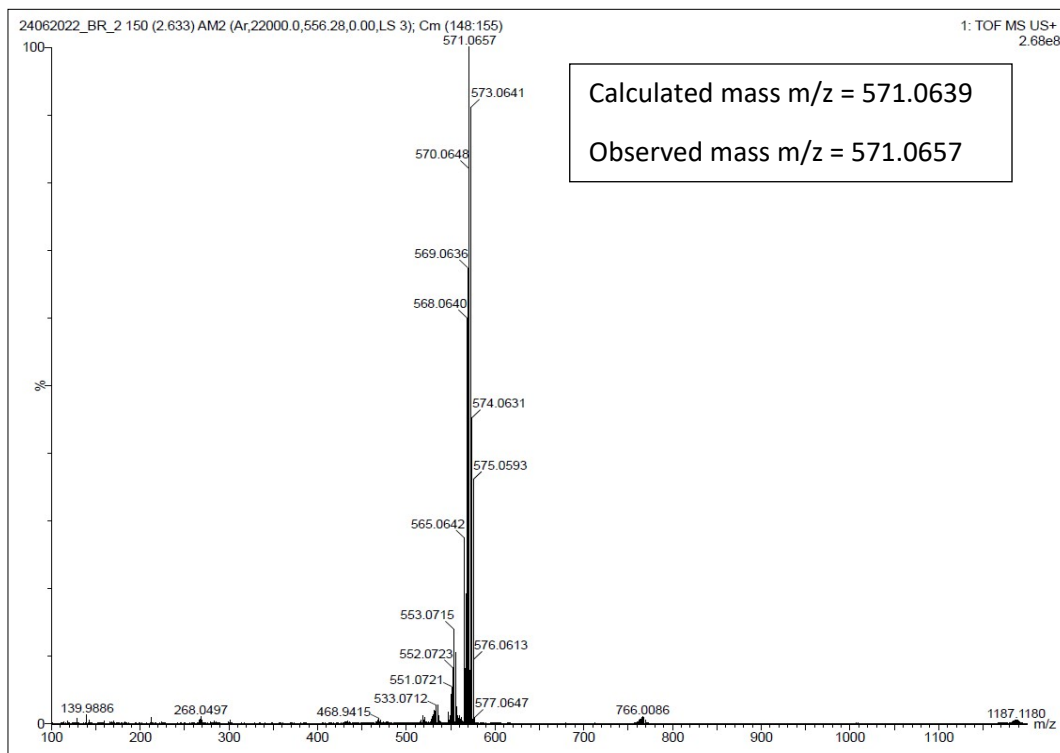
¹H NMR of complex RuL3:

¹³C NMR of complex RuL3:

Signature SIF VIT VELLORE
RuL3

Peaks: 158.75, 153.18, 148.33, 148.02, 145.44, 142.90, 141.28, 139.37, 139.02, 136.06, 135.45, 131.73, 130.80, 129.63, 128.45, 126.97, 123.65, 116.88, 105.60, 103.77, 87.11, 84.86, 77.35, 77.03, 76.72, 29.71, 22.46, 22.34, 19.20

ppm axis: 200 180 160 140 120 100 80 60 40 20 0 ppm

^{19}F NMR of complex RuL3:

Signature SIF VIT VELLORE
RuL3

-69.19
-71.07

0   -20   -40   -60   -80   -100   -120   -140   -160   -180   ppm

³¹P NMR of complex RuL3:

Signature SIF VIT VELLORE
RuL3

-131.03
-135.43
-139.81
-144.20
-148.60
-152.98
-157.38

100   50   0   -50   -100   -150   -200   ppm

HRMS spectrum of complex RuL3:

Calculated mass m/z = 632.9818

Observed mass m/z = 632.9837

^{1}H NMR of complex RuL4:

^{13}C NMR of complex RuL4:

Signature SIF VIT VELLORE
RuL4

159.40, 158.84, 148.75, 144.39, 141.39, 140.86, 136.11, 131.76, 129.35, 128.44, 128.30, 126.12, 125.27, 123.65, 118.01, 105.89, 103.89, 87.00, 86.95, 84.73, 77.35, 77.03, 76.71, 31.37, 22.33, 21.15, 19.12

^{19}F NMR of complex RuL4:

Signature SIF VIT VELLORE
RuL4

-69.19
-71.08

0   -20   -40   -60   -80   -100   -120   -140   -160   -180   ppm

^{31}P NMR of complex RuL4:

Signature SIF VIT VELLORE
RuL4

-135.43
-139.81
-144.20
-148.60
-152.98
-157.38

100   50   0   -50   -100   -150   -200   ppm

HRMS spectrum of complex RuL4:

24062022_BR_4 149 (2.616) AM2 (Ar,22000.0,556.28,0.00,LS 3); Cm (146:152)   1: TOF MS US+
2.20e8

Calculated mass m/z = 598.0584

Observed mass m/z = 598.0598

¹H NMR of complex RuL5:

Signature SIF VIT VELLORE
RuL5

^{13}C NMR of complex RuL5:

Signature SIF VIT VELLORE
RuL5

158.57, 148.29, 148.03, 146.60, 142.68, 141.14, 139.51, 138.51, 136.33, 135.54, 135.39, 133.49, 130.91, 129.65, 129.55, 128.29, 127.05, 122.01, 105.60, 103.49, 87.03, 84.92, 77.35, 77.03, 76.71, 31.30, 22.43, 22.34, 19.16

^{19}F NMR of complex RuL5:

Signature SIF VIT VELLORE
RuL5

-69.19
-71.08

0   -20   -40   -60   -80   -100   -120   -140   -160   -180   -200   ppm

^{31}P NMR of complex RuL5:

Peaks: -135.43, -139.81, -144.20, -148.59, -152.98

HRMS spectrum of complex RuL5:

Calculated mass m/z = 587.0343
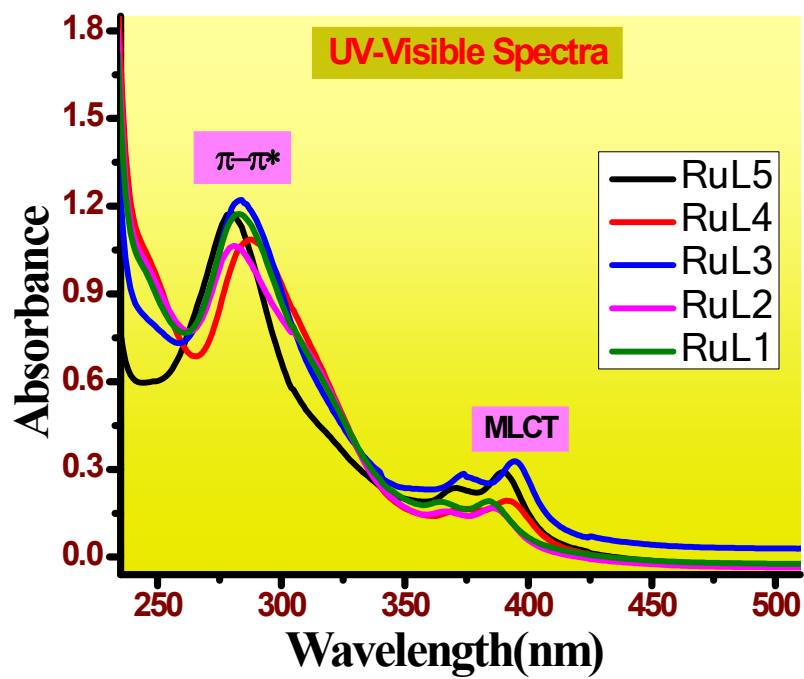
Observed mass m/z = 587.0352

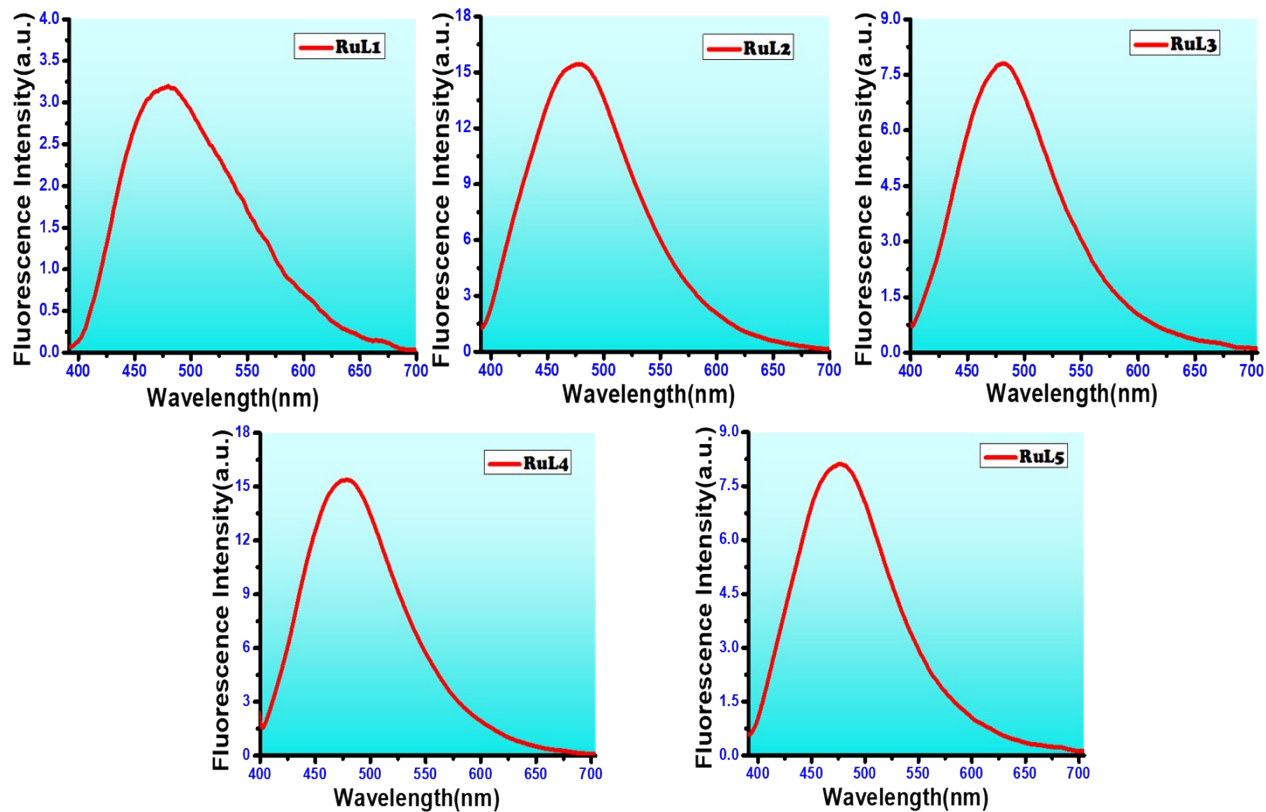**Fig. S1: Absorption spectra of complexes RuL1-RuL5 in 10% DMSO solutions (3 x 10^{-5} M)**

Fig. S2: Emission spectra of complexes RuL1–RuL5 in 10% DMSO-water (concentration = 3 x 10^{-5} M, λex = 300 nm).
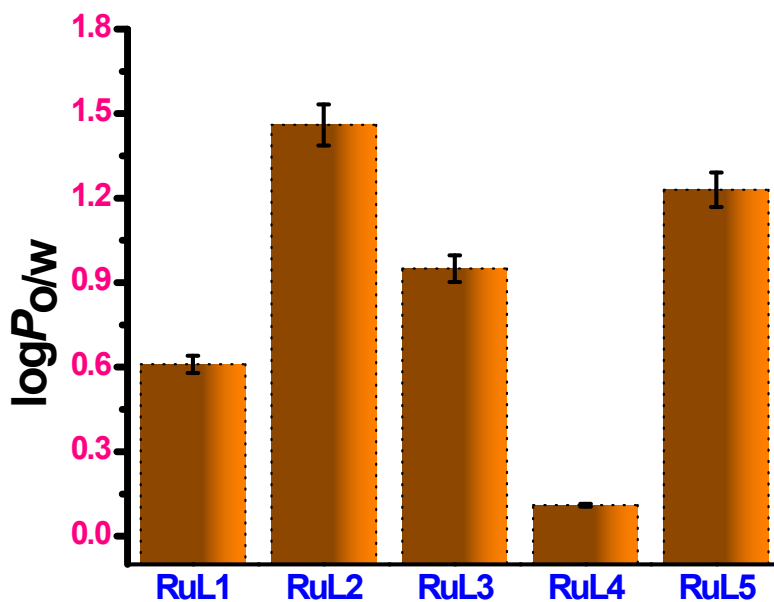
Fig. S3: Representative diagram of lipophilicity of RuL1–RuL5 showed by a bar diagram displaying comparative log $P_{o/w}$ values of complexes in Octanol–water system, where error bars in the graph specify the standard deviation in measurement.
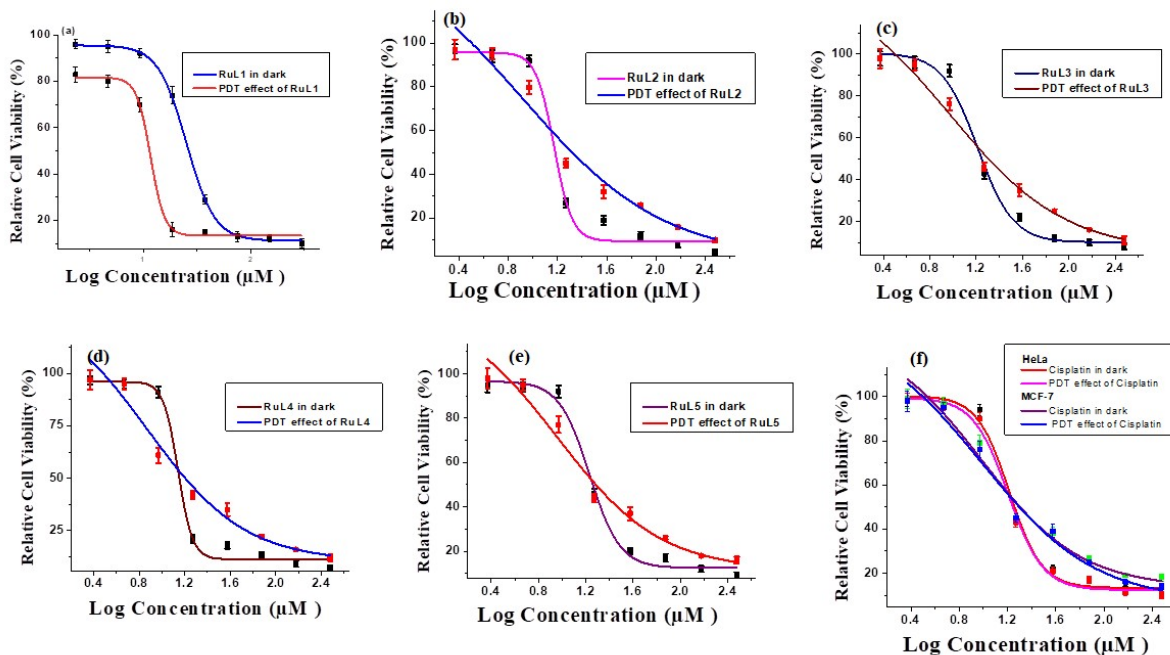

Fig. S4: Cytotoxicity study of (a-e) complex RuL1-RuL5 against HeLa cell line under dark and in presence of light (f) Cisplatin against HeLa and MCF-7 cell line under dark and in presence of light
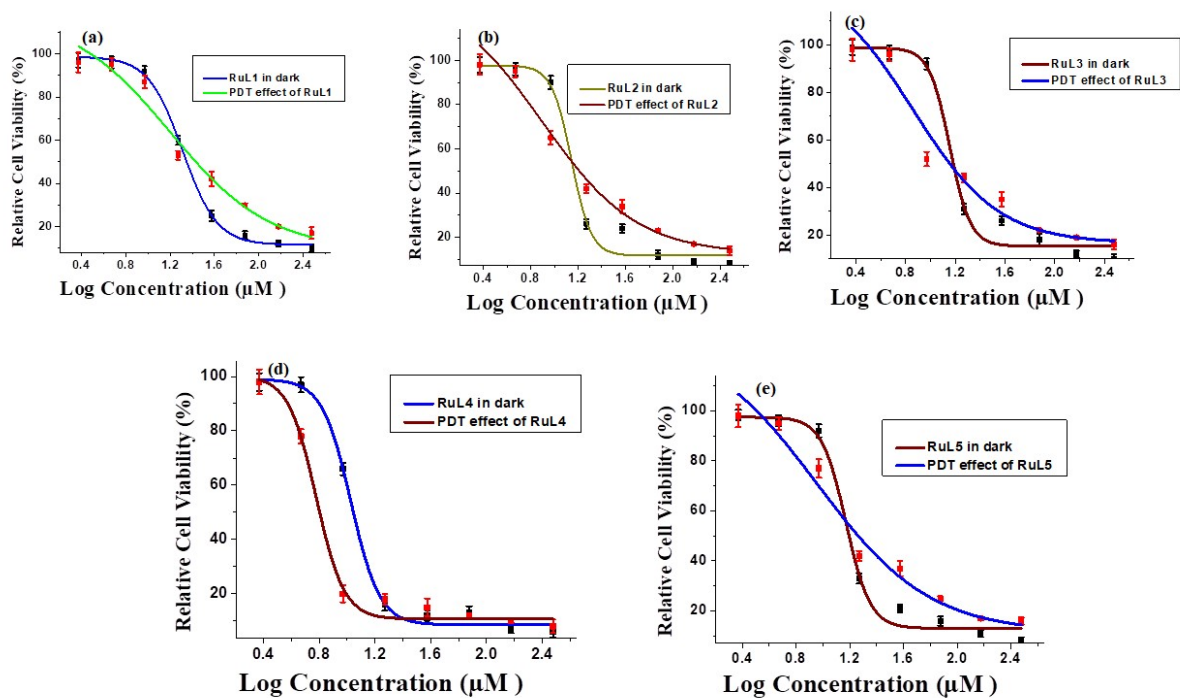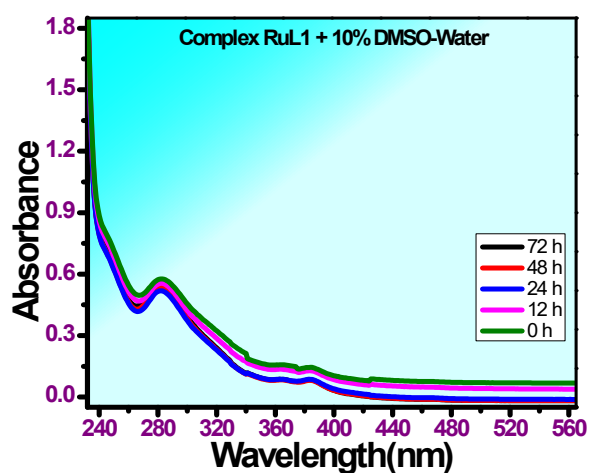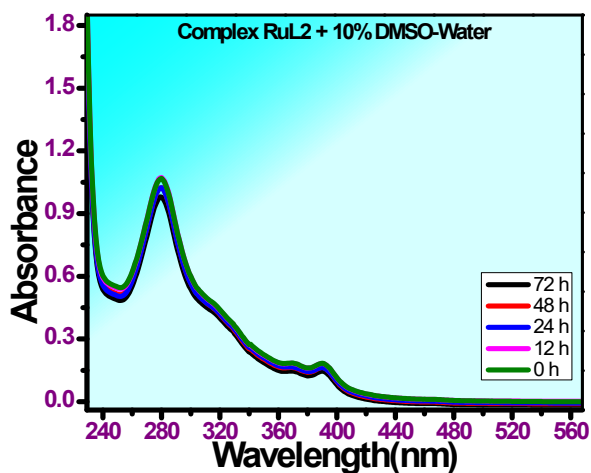
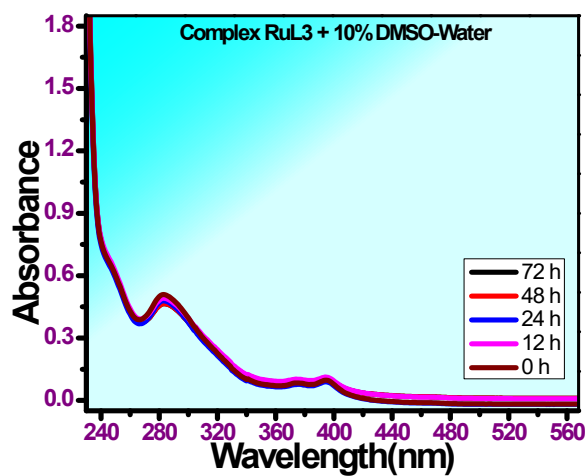**Fig. S5: Cytotoxicity study of (a-e) complex RuL1-RuL5 against MCF-7 cell line under dark and in presence of light**

(a)

(b)

(c)

(d)

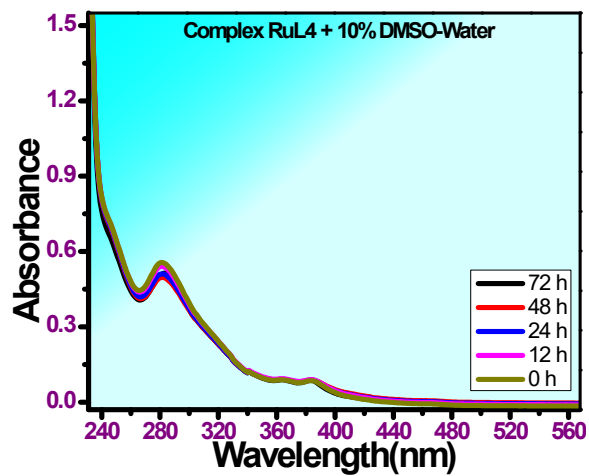**(e)**

Fig. S6: Stability of all five complexes (RuL1-RuL5) in 10% DMSO media.

(a)

(b)
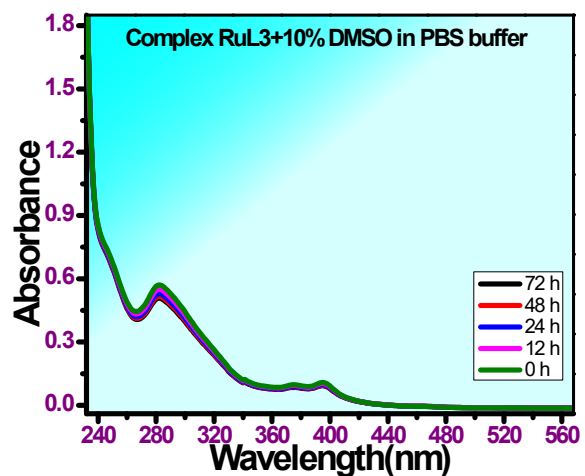
(c)
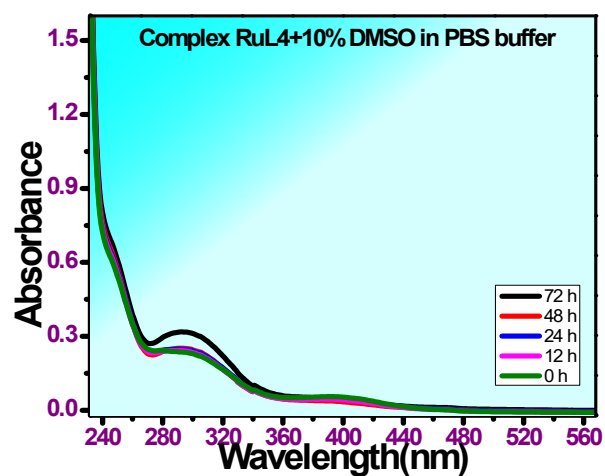
(d)
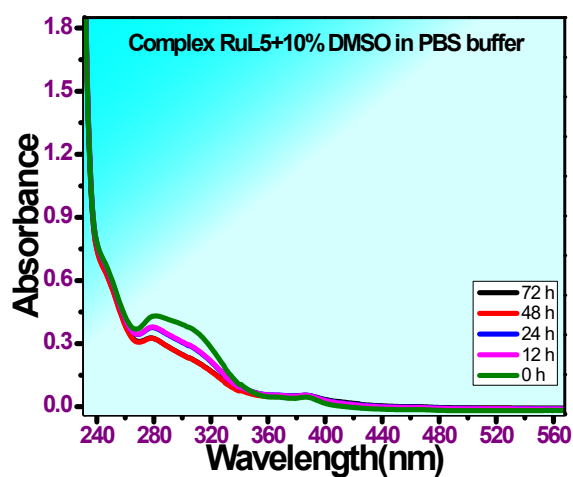
(e)

Fig. S7: Stability of all five complexes (RuL1-RuL5) in 10% DMSO-PBS buffer media.
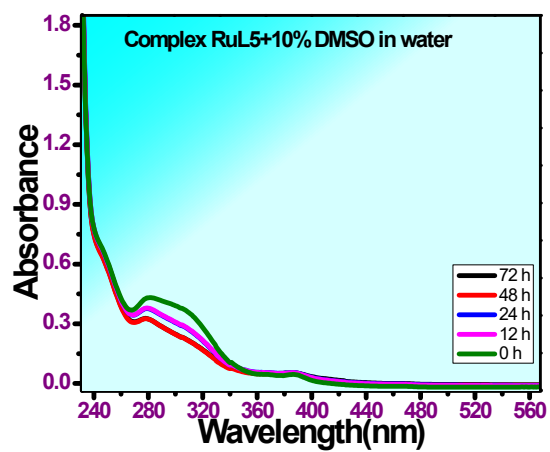
(a)

(b)

(c)

(d)

(e)

33
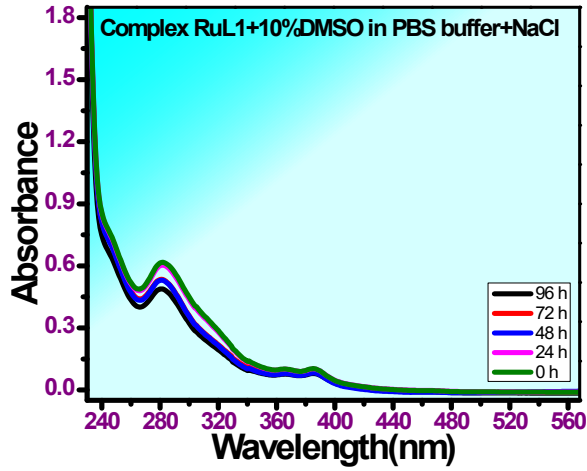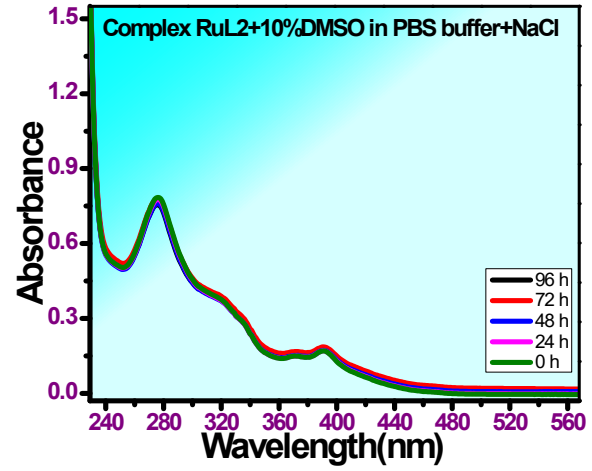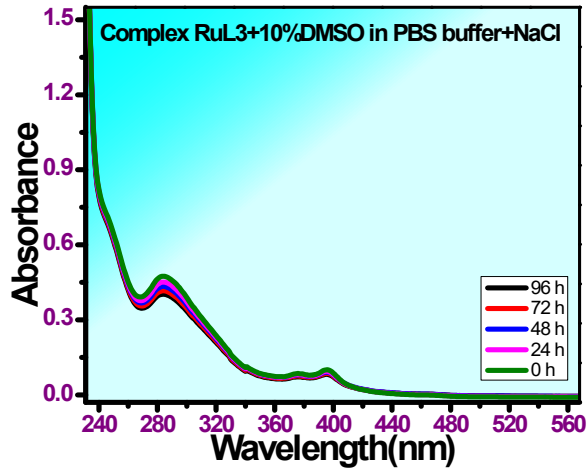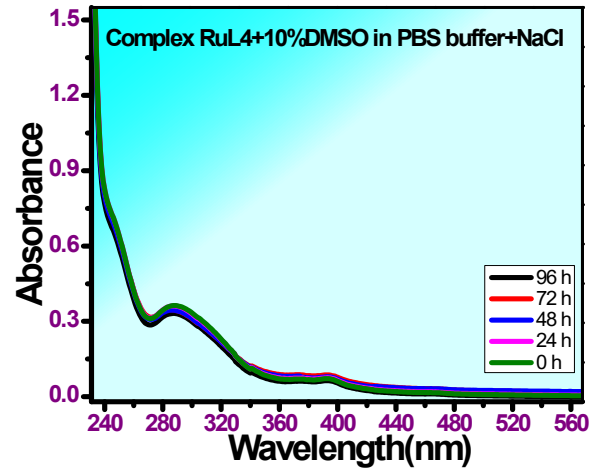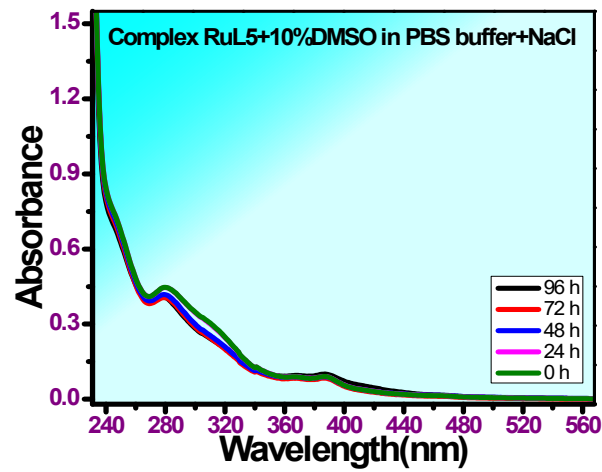
Fig. S8: Stability of all five complexes (RuL1-RuL5) in 10% DMSO-PBS buffer media in presence of NaCl.
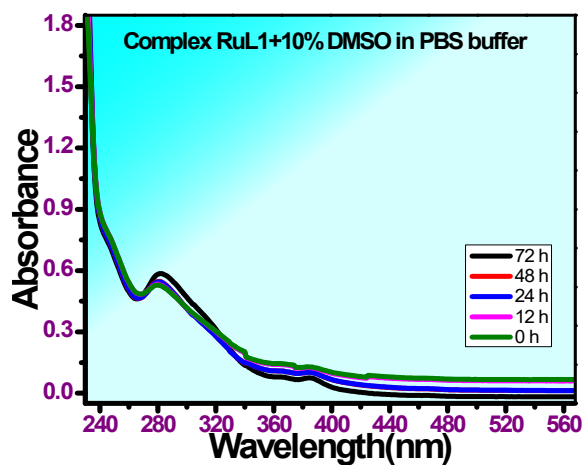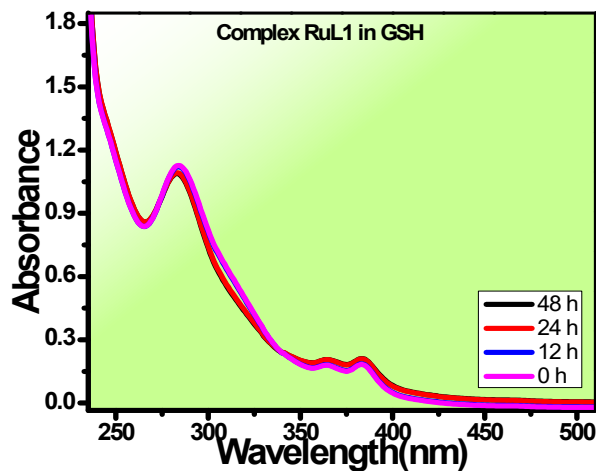

(a)
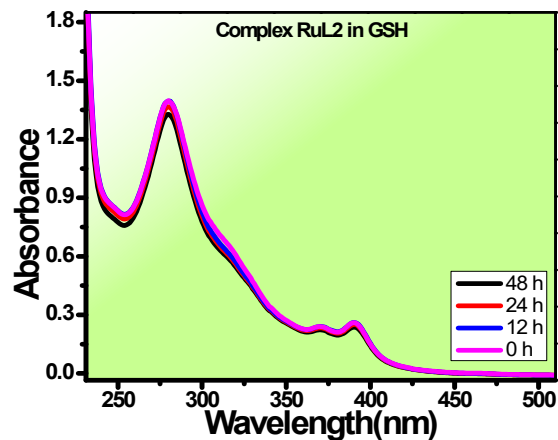
(b)
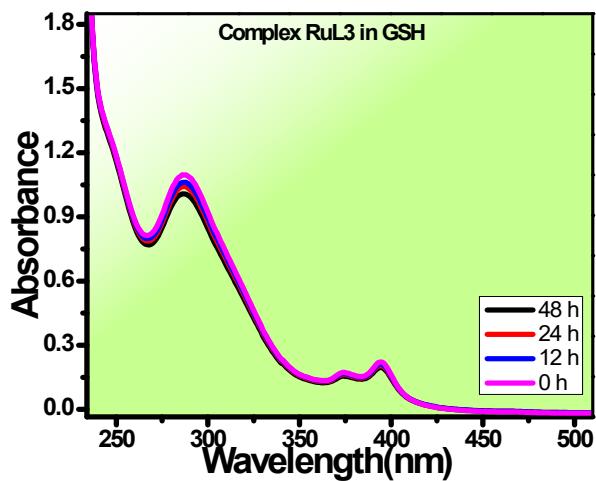
(c)
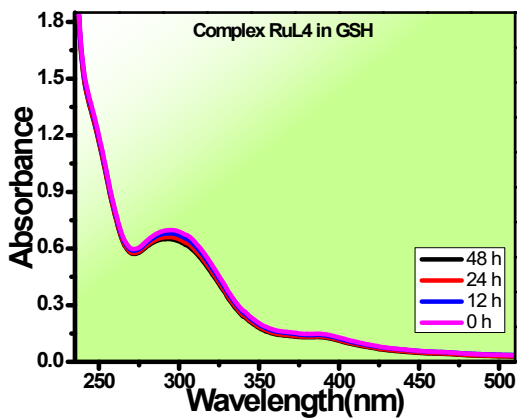
(d)
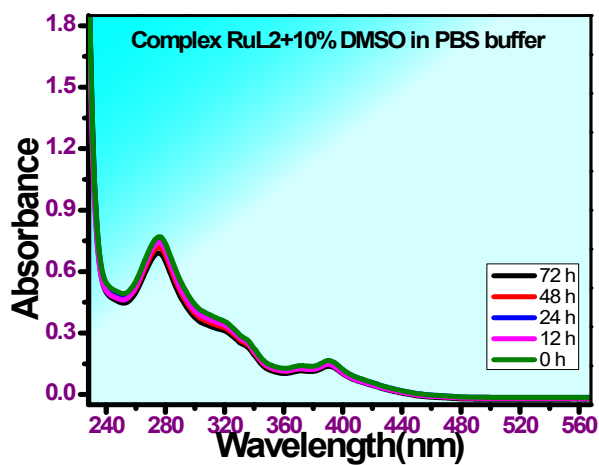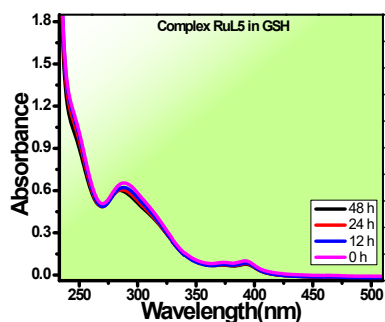
(e)

Fig. S9: Stability of all five complexes (RuL1-RuL5) in 1mM 1mM GSH media
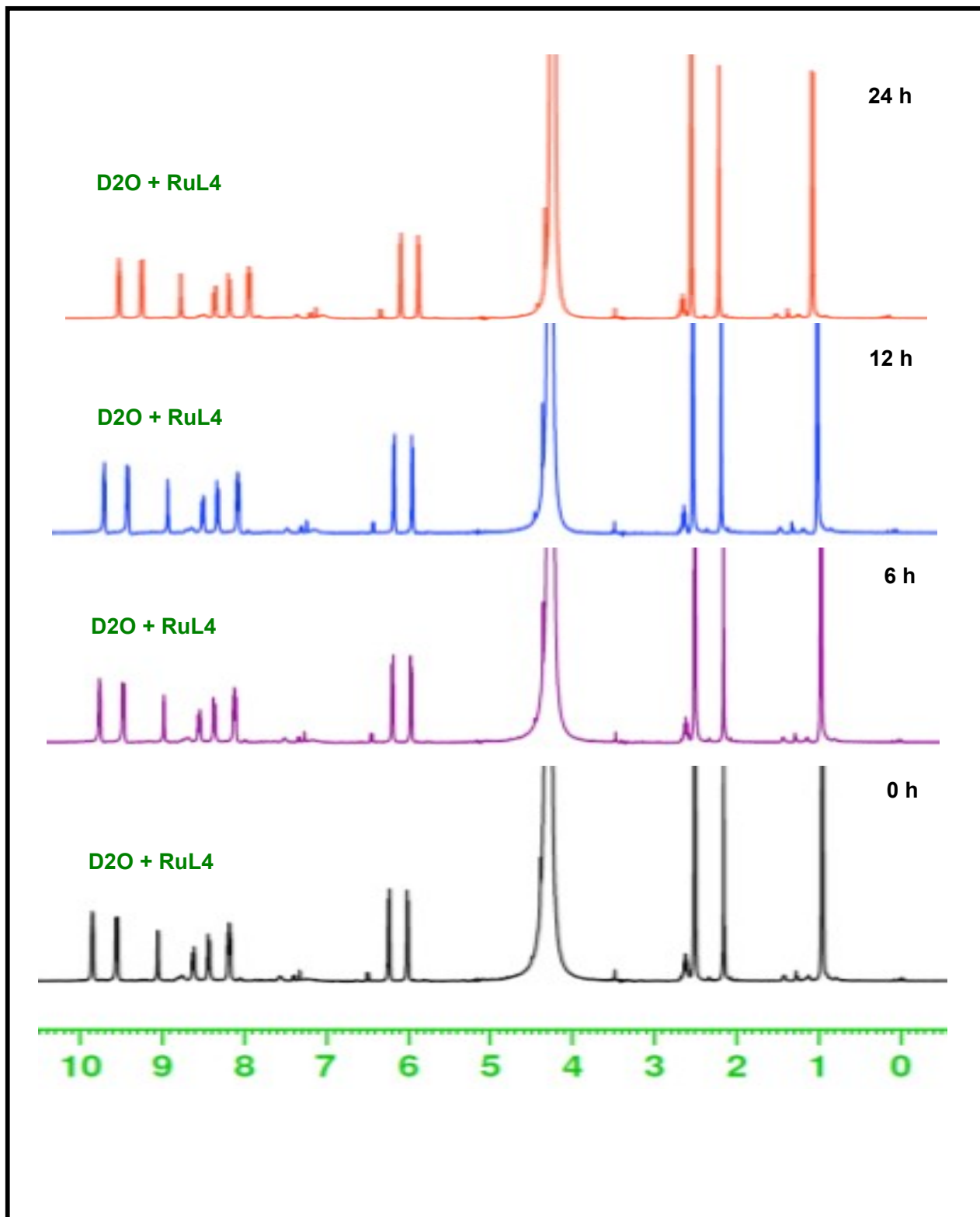
Fig. S10 Stability study of complex **RuL4** in presence of DMSO-D$_2$O *via* ^{1}H NMR.
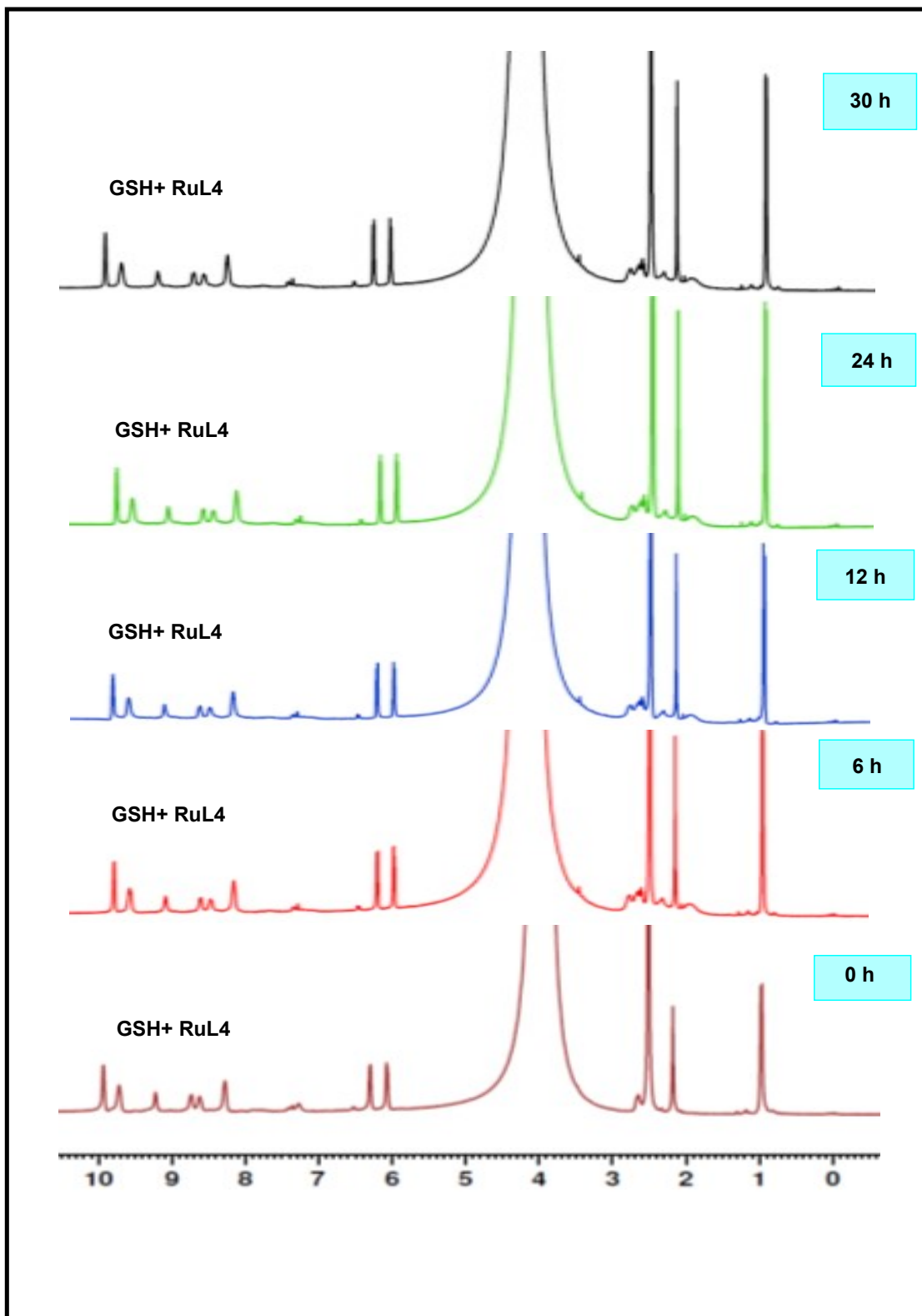
Fig. 11 Stability study of complex **RuL4** in presence of reduced L-glutathione and water *via* ¹H NMR. t = 0 h, stands for the spectra recorded immediately after dissolving reduced L-glutathione and complex.
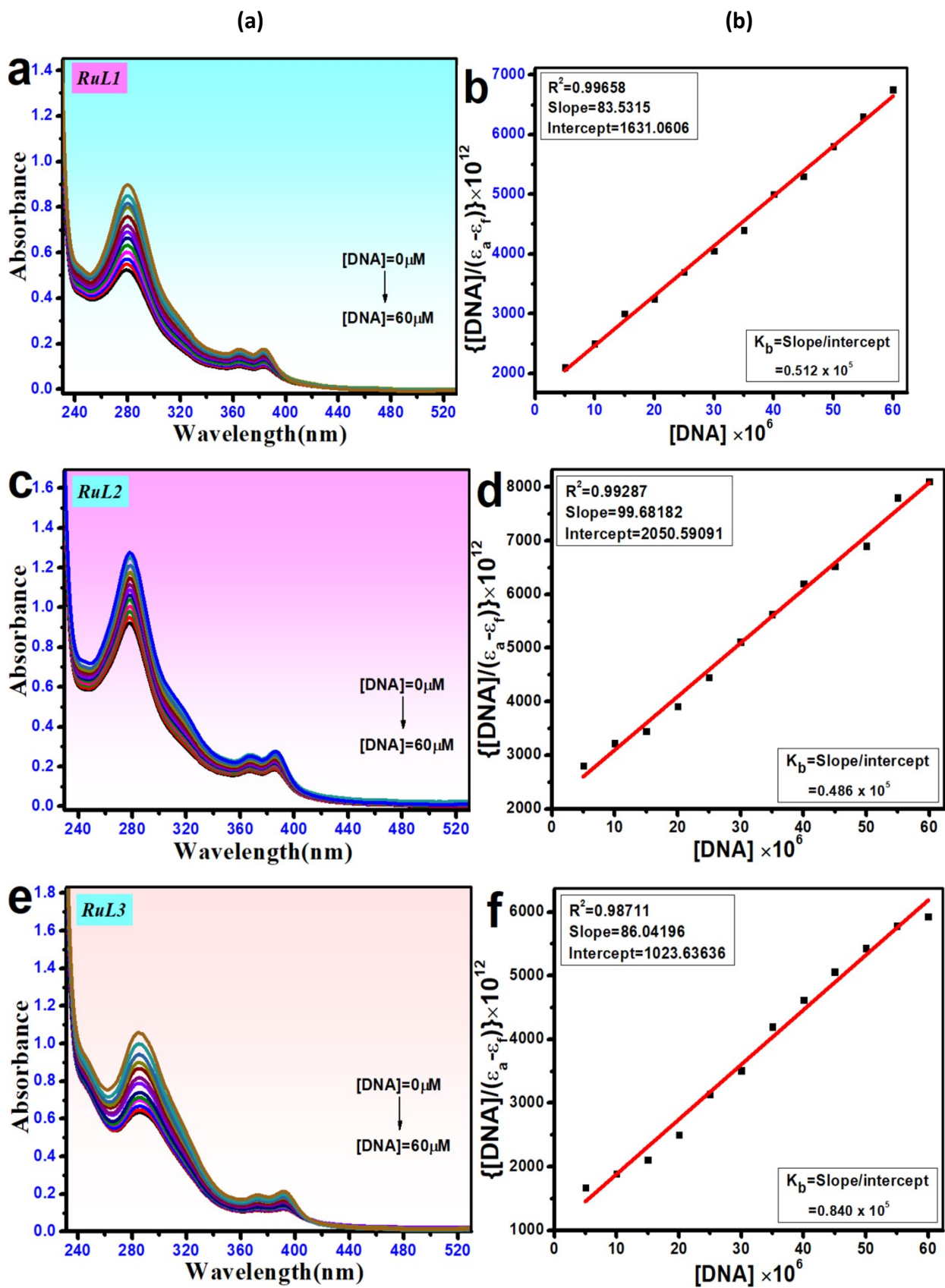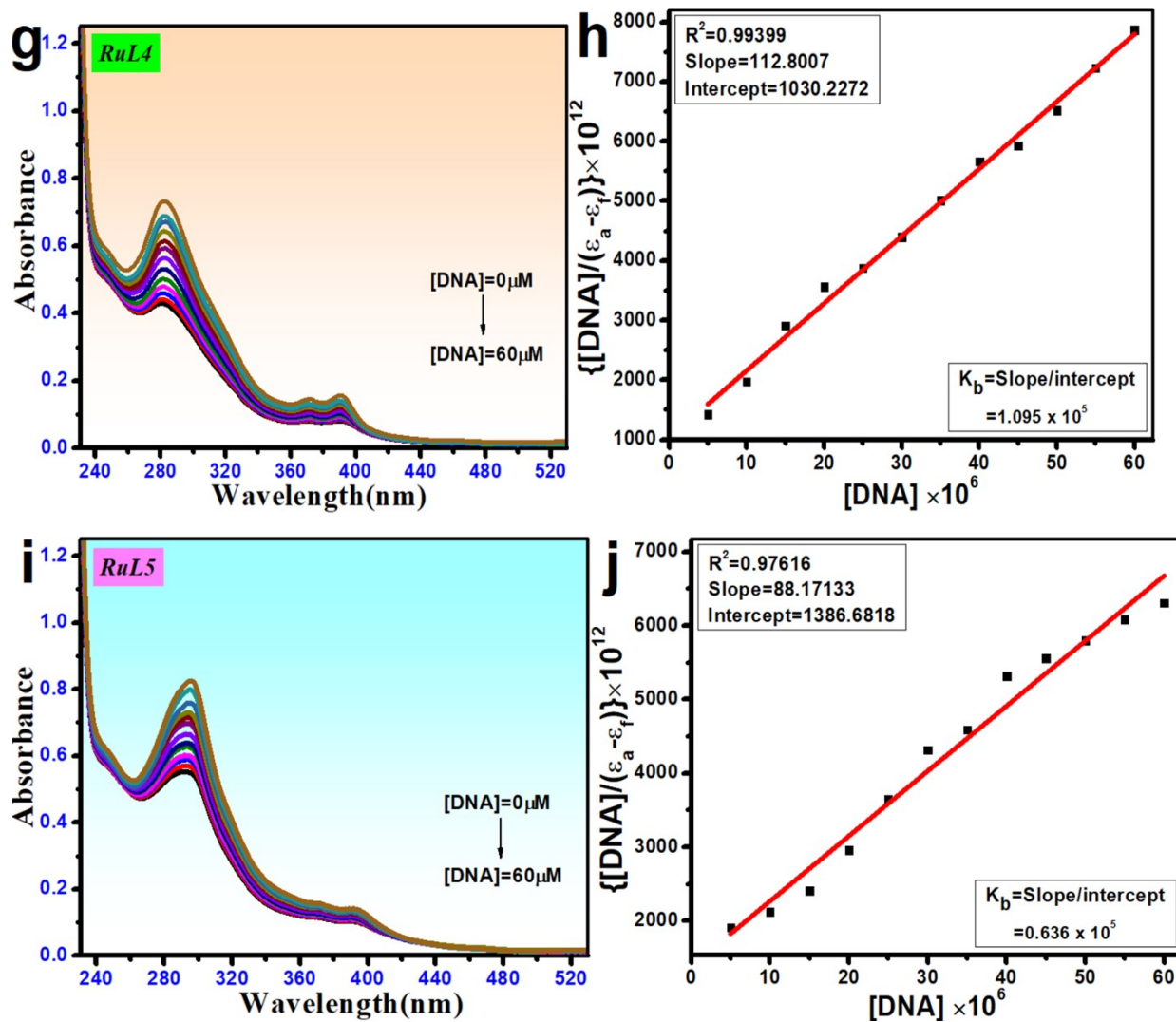
Fig. S12: DNA binding plots of all five complexes [(a), (c), (e), (g), and (i)]. [DNA]/(ε_a-ε_f) vs. [DNA] linear plots of all five complexes [(b), (d), (f), (h), and (j)].

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)

(k)

(l)

(m)

(n)

(o)
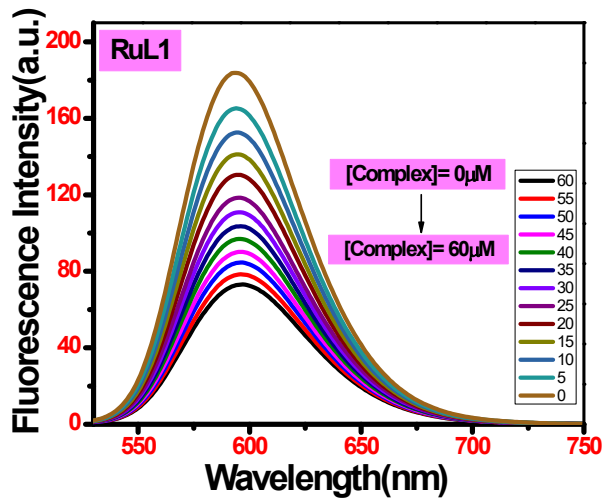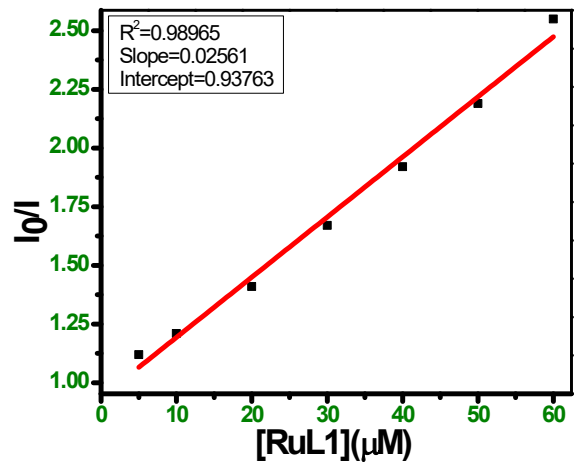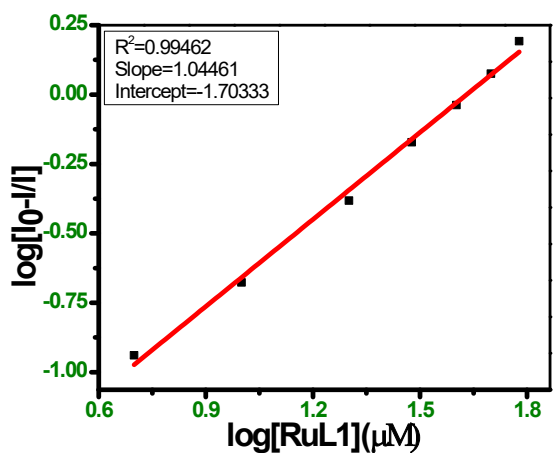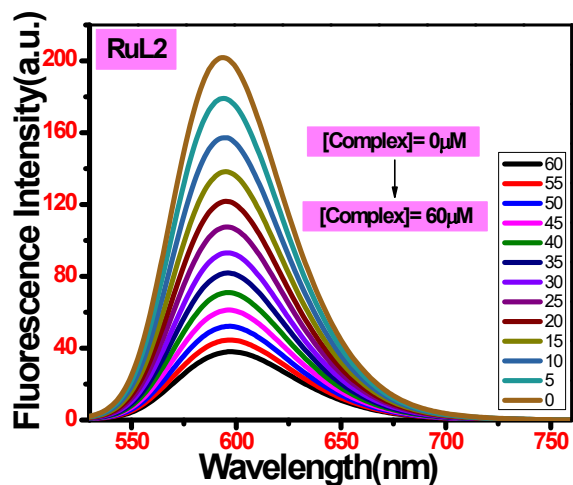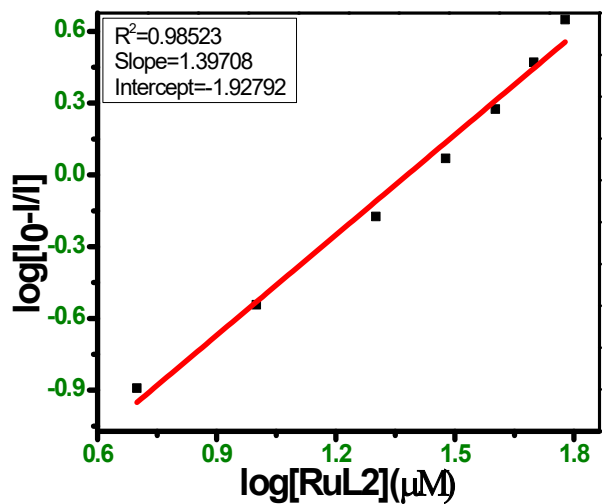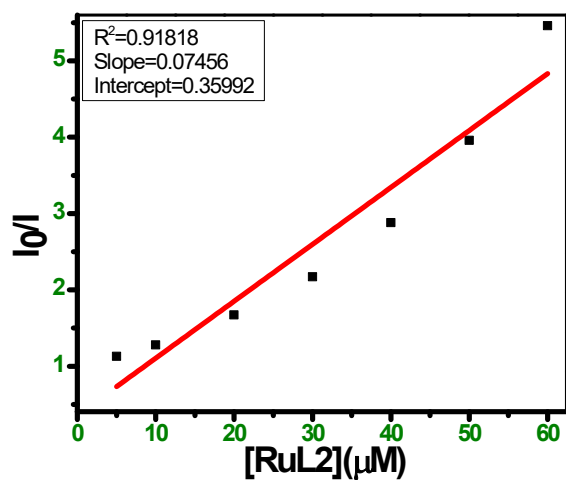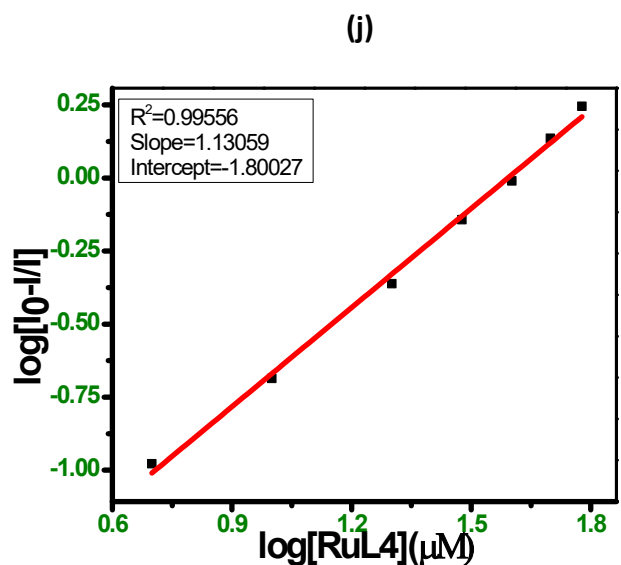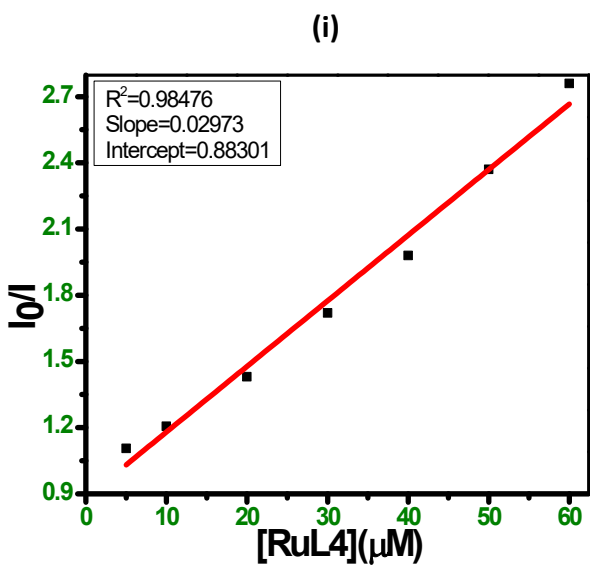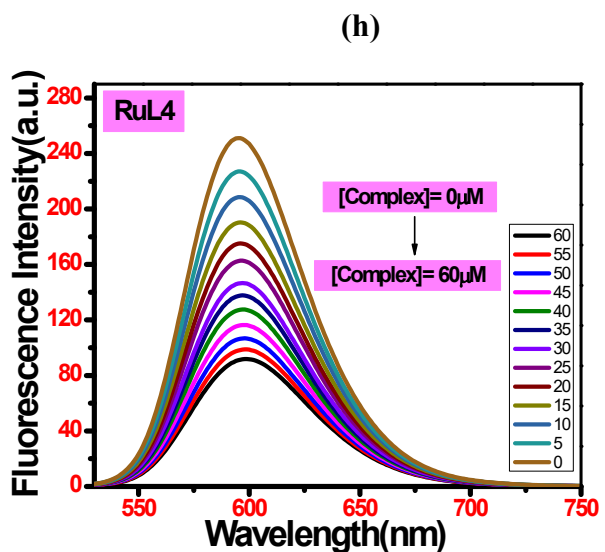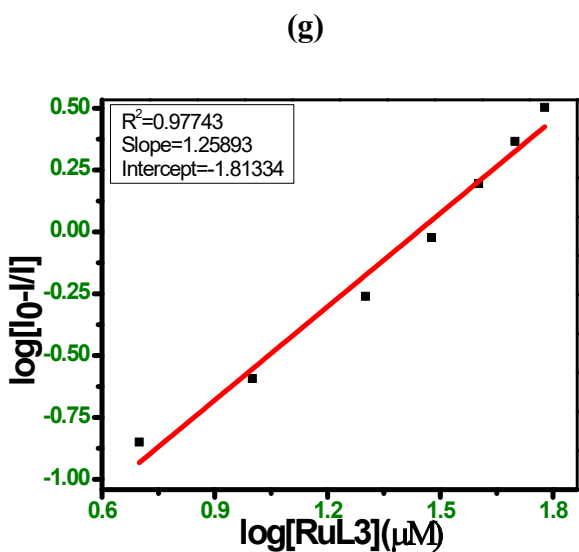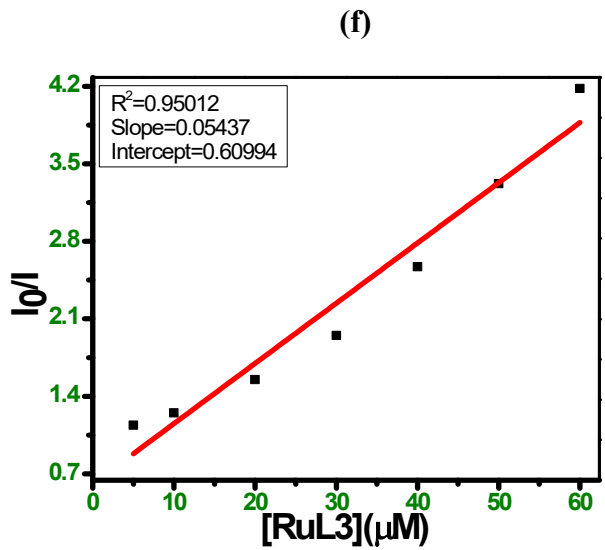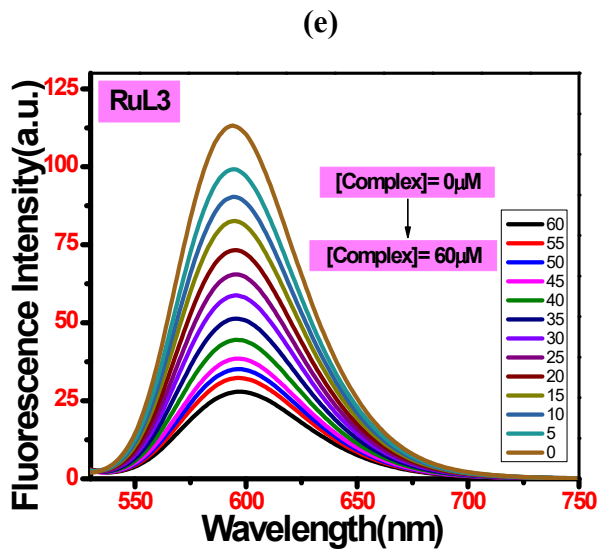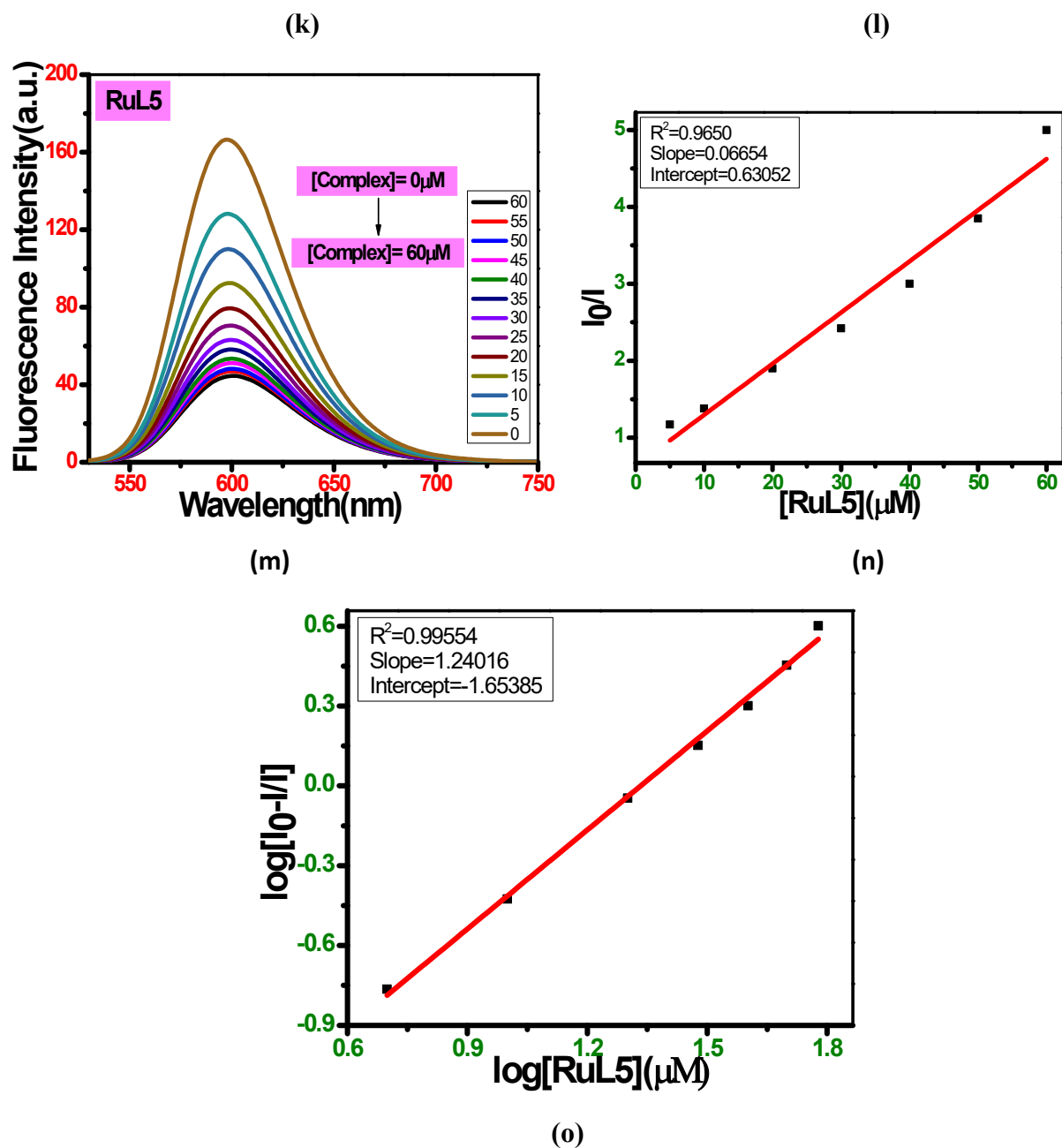
Fig. S13: Interaction of all five complexes with EtBr [(a), (d), (g), (j), and (m)]. Stern-Volmer Plot of I_0/I vs. concentration of complex [(b), (e), (h), (k), and (n)]. Scatchard Plot of $\log([I_0-I]/I)$ vs. $\log[Complex]$ for EtBr in the presence of metal complexes [(c), (f), (i), (l), and (o)].

g RuL3

Fluorescence Intensity(a.u.)

[Complex]=0μM

[Complex]=60μM

Wavelength(nm)

h
R^2=0.97246
Slope=0.11297
Intercept=0.33139

I_0/I

[RuL3] μM

i
R^2=0.99581
Slope=1.36367
Intercept=-1.75263

$\log[(I_0-I)/I]$

$\log$[RuL3] μM

j RuL4

Fluorescence Intensity(a.u.)

[Complex]=0μM

[Complex]=60μM

Wavelength(nm)

k
R^2=0.96853
Slope=0.09611
Intercept=0.26701

I_0/I

[RuL4] μM

l
R^2=0.99451
Slope=1.33751
Intercept=-1.78054

$\log[(I_0-I)/I]$

$\log$[RuL4] μM
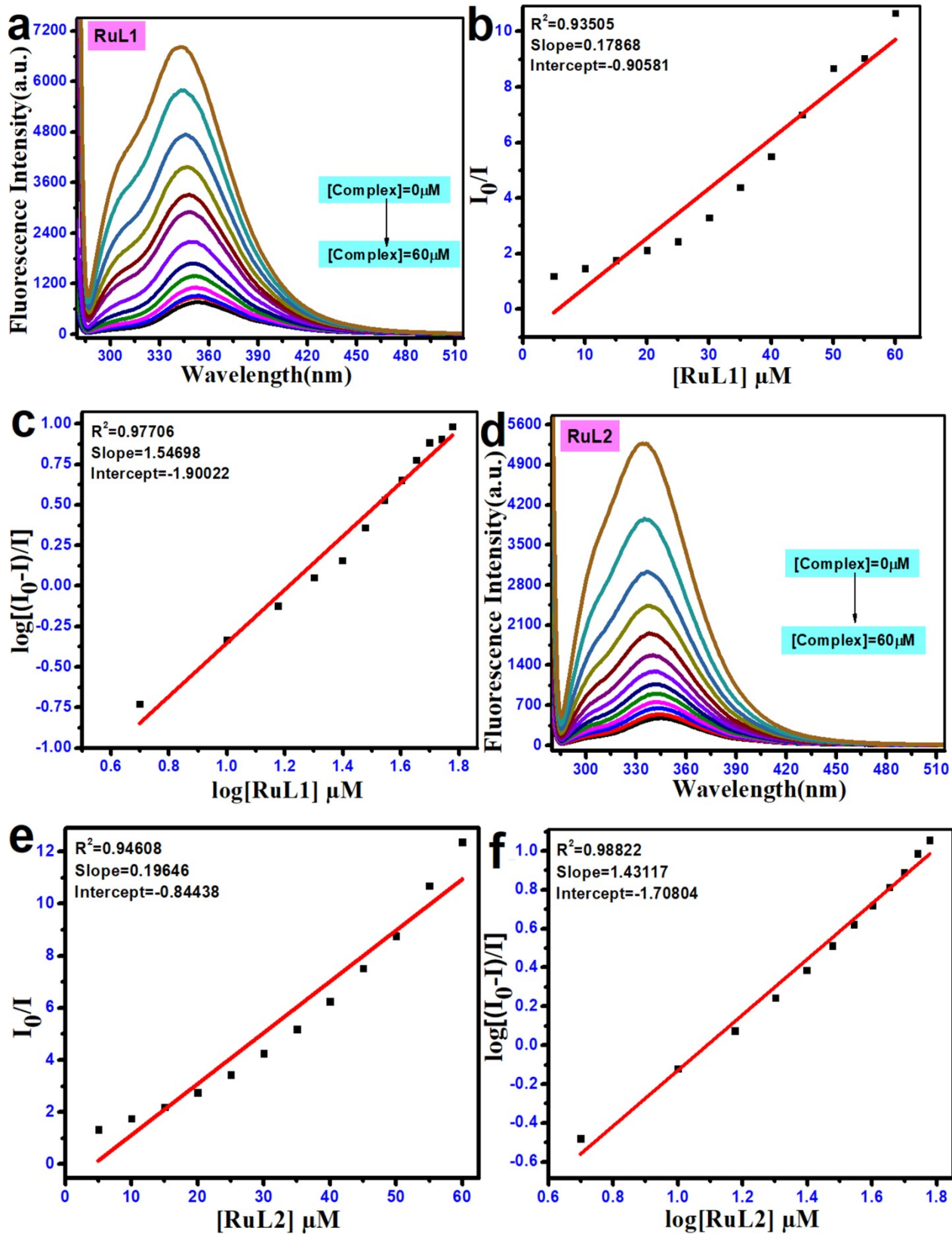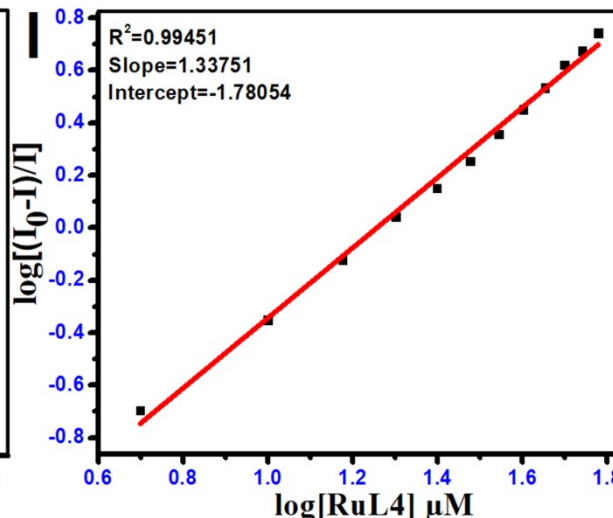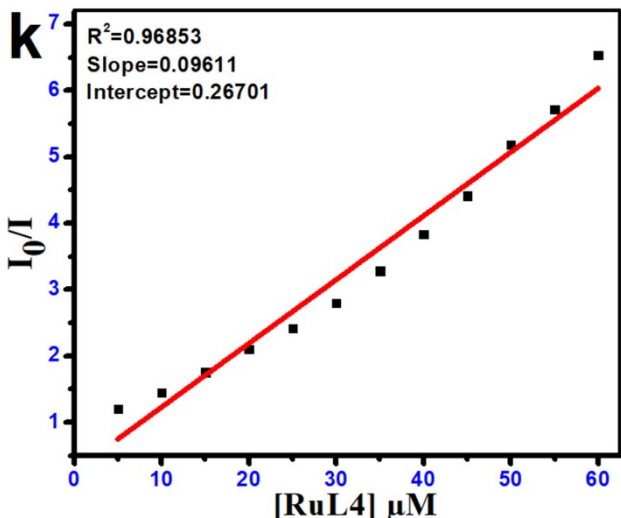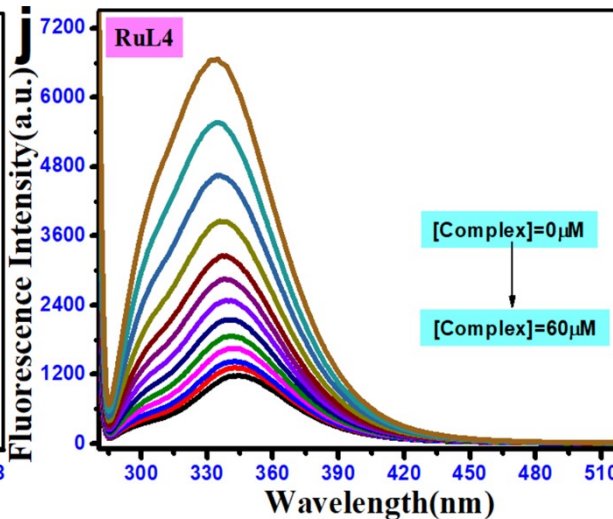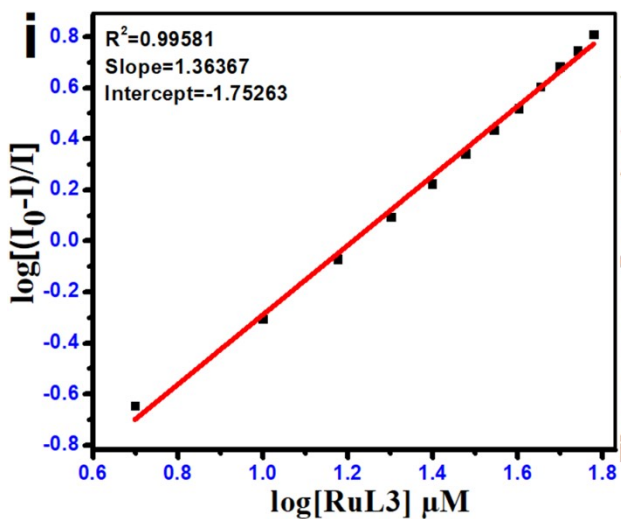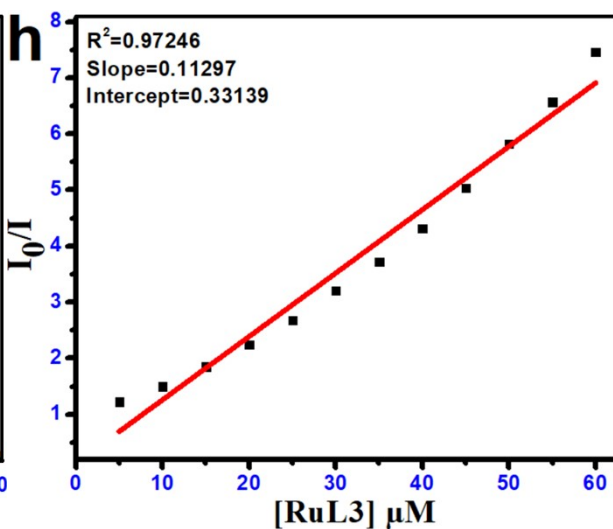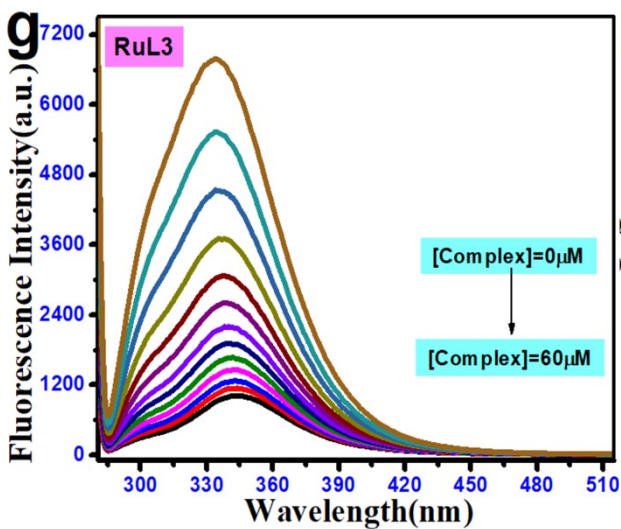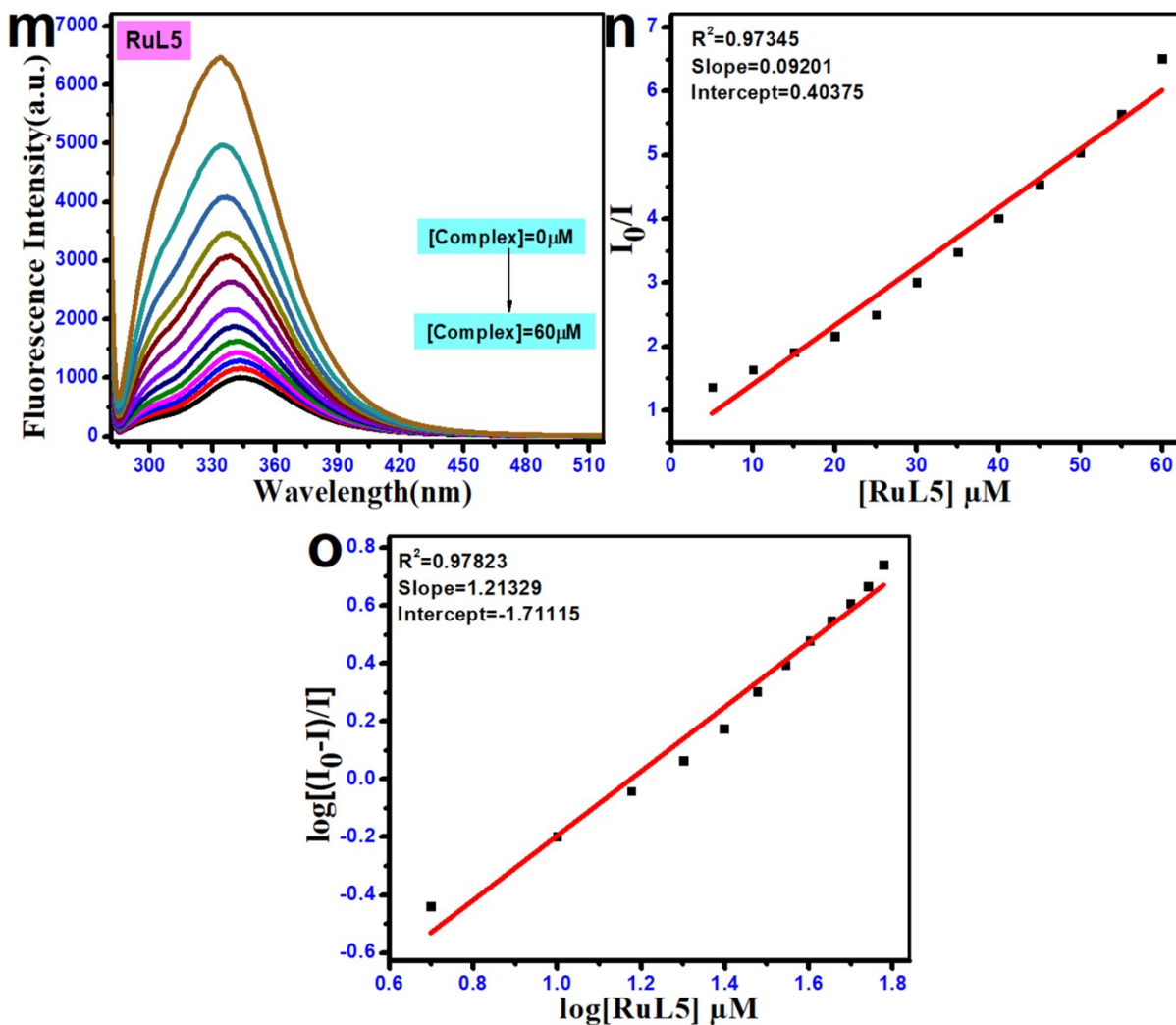
Fig. S14: Interaction of all five complexes with HSA [(a), (d), (g), (j), and (m)]. Stern-Volmer Plot of I_0/I vs. concentration of complex [(b), (e), (h), (k), and (n)]. Scatchard Plot of $\log[(I_0-I)/I]$ vs. log[Complex] for HSA in the presence of metal complexes [(c), (f), (i), (l), and (o)].

Experimental Section

DNA binding study

Electronic absorption spectroscopy was employed to study the binding capacity of the complexes with calf-thymus DNA (Ct-DNA) and competitive binding assay as studied using ethidium bromide (EtBr) as quencher by fluorescence spectroscopy.

UV–visible studies[1]

DNA binding assay was carried out by using complexes **[RuL1-RuL5]** in Tris-HCl buffer (5 mM Tris-HCl in water, pH 7.4) in aqueous medium. The concentration of CT-DNA was calculated from its absorbance intensity at 260 nm and its known molar absorption coefficient value of 6600 M^{-1} cm^{-1}. Equal amount of DNA was added in both the sample and reference in cuvettes. Titration was carried out by increasing concentration of CT-DNA. On the eve of each measurement, sample was equilibrated with CT-DNA for about 5 min and then absorbance of the complex was measured. The intrinsic DNA binding constant (K_b) was calculated using the equation (i):

$$\frac{[DNA]}{(\varepsilon_a - \varepsilon_f)} = \frac{[DNA]}{(\varepsilon_b - \varepsilon_f)} + \frac{1}{K_b(\varepsilon_a - \varepsilon_f)} \mathsf{L} \mathsf{L} \ (i)$$

Where [DNA] is the concentration of DNA in the base pairs, ε_a is the apparent extinction coefficient observed for the complex, ε_f corresponds to the extinction coefficient of the complex in its free form, and ε_b refers to the extinction coefficient of the complex when fully bound to DNA. The resultant data were plotted using Origin Lab, version 8.5 to obtain the [DNA]/(ε_a-ε_f) vs. [DNA] linear plot. The ratio of the slope to intercept from the linear fit gave the values of the intrinsic binding constants (K_b).

Photophysical study

UV and Fluorescence study of all these Ru(II) complexes were executed in 10 % DMSO solution. Then the fluorescence quantum yields (Φ) were calculated by applying the comparative William's method which involves the use of well-characterized standard with known quantum

yield value using 10% DMSO solution.[2] Quinine sulphate was used as a standard. Quantum yield was calculated according to the equation (ii):

$$\varphi = \varphi_R \times \frac{I_S}{I_R} \times \frac{OD_R}{OD_S} \times \frac{\eta_S}{\eta_R} \cdots\cdots\cdots(ii)$$

Where, φ = quantum yield, I = peak area, OD = absorbance at λmax, η = refractive index of solvent (s) and reference (R). Here, we have used quinine sulphate as a standard for calculating the quantum yield.

Ethidium bromide displacement assay

The ethidium bromide (EtBr) displacement assay was conducted to illustrate the mode of binding between the potent compounds with DNA.[3] The apparent binding constant (K_{app}) of all the Ru(II) complexes to CT-DNA were calculated using ethidium bromide (EtBr) as a spectral probe in 5 mM Tris-HCl buffer (pH 7.4). EtBr do not show any fluorescence in its free state as its fluorescence is quenched by the solvent molecules. Nevertheless, its fluorescence intensity was radially increases with increase the concentration of CT-DNA, which suggested the intercalative mode of binding of EtBr with DNA grooves. The fluorescence intensity was found to decrease with further increase in concentration of the complexes. According to the displacement theory, it can be said that the complexes displaced EtBr from CT-DNA grooves and then bound to the DNA base pairs. The values of the apparent binding constant (K_{app}) were obtained by using the equation (iii):

$$K_{app} \times [Complex]_{50} = k_{EtBr} \times [EtBr]\cdots\cdots\cdots(iii)$$

Where K_{EtBr} is the EtBr binding constant (K_{EtBr} = 1.0 x 10^7 M^{-1}), and [EtBr] = 8 x 10^{-6} M. Stern-Volmer equation was followed for quantitative determination of the Stern-Volmer quenching constant (K_{SV}).[4] Origin (8.5) software was used to plot the fluorescence data to obtain linear plot of I_0/I vs. [complex]. The value of K_{SV} was calculated from the following equation.

$$I_0/I = 1 + K_{SV}[Q]\mathsf{L} \mathsf{L} \ (iv)$$

Where I_0 = fluorescence intensity in absence of complex and I = fluorescence intensities in presence of complex of concentration [Q].

Protein binding studies

We are acquainted with the fact that serum albumin proteins are the main component. It is well known in blood plasma proteins and plays important roles in drug transport and metabolism, interaction of the drug with human serum albumin (HSA) was studied from tryptophan emission quenching experiment.[5] Initially, HSA solution (2 x 10^{-6} M) was prepared in Tris-HCl/NaCl buffer. The aqueous solutions of the complexes were subsequently added to HSA solution with gradual increase of their concentrations. After each addition, the solutions were shaken slowly for 5 min before recording the fluorescence at a wavelength of 280 nm (λ_{ex} = 280 nm). A gradual decrease in fluorescence intensity of HSA at λ = 345 nm was observed upon increasing the concentration of complex, which confirmed that the interaction between the complex and HSA was happened. Stern-Volmer equation was employed to quantitatively determine the quenching constant (K_{HSA}). Origin Lab, version 8.5 was used to plot the emission spectral data to obtain linear plot of I_0/I vs. [complex] using the equation (v) given below:

$$I_0/I = 1 + K_{HSA}[Q] = 1 + k_q \tau_0 [Q] \cdots (v)$$

Where I_0 is the fluorescence intensity of HSA in absence of complex and I indicates the fluorescence intensity of HSA in presence of complex of concentration [Q], τ_0 = lifetime of the tryptophan in HSA found as 1 x 10^{-8} and k_q is the quenching constant. Scatchard equation (vi) gives the binding properties of the complexes.[6] Where K = binding constant and n = number of binding sites.

$$\log(I_0 - I/I) = \log K + n \log [Q] \; \llcorner \; \llcorner \; (vi)$$

Conductivity measurement[7]

For authenticating the interaction of the complexes with DMSO and aqueous DMSO, conductivity of the prepared complexes were performed using conductivity-TDS meter-307 (Systronics, India) and cell constant 1.0 cm^{-1}. Rate of conductivity was also estimated in different pH medium. Time dependent conductivity measurement was also carried out.

n-Octanol–water partition coefficient (log $P_{o/w}$)[8]

The log $P_{o/w}$ of the Ru(II) complexes were adhering to shake flask method using the previously published procedure. A known amount of each Ru(II) complexes was suspended in water (pre-saturated with n-octanol) and shaken for 48 h on an orbital shaker. To allow the phase separation, the solution was centrifuged for 10 min at 3000 rpm. To obtain the partition coefficient, different ratios (0.5: 1, 1: 1, and 2: 1) of the saturated solutions were shaken with pre-saturated n-octanol for 20 min on an orbital shaker and followed the same procedure. Aliquots of the aqueous and octanol layers were pipetted out separately and the absorbances were measured with UV-Vis spectrophotometer using proper dilution. Each set was performed in triplicate, concentration of the substances in each layer was calculated using the respective molar extinction coefficients and the partition coefficient (log $P_{o/w}$) values were obtained from the ratio.

Singlet oxygen ($^1O_2$) quantum yield determination [9]

The singlet oxygen ($^1O_2$) quantum yields of the complex **RuL4** at ambient temperature in DMSO were determined using visible light (400–700 nm) for photosensitization. The $^1O_2$ quantum yields were determined by monitoring the photooxidation of DPBF after sensitization by the complex. DPBF is a convenient acceptor because it absorbs in the region where the dye is transparent and rapidly scavenges singlet oxygen to generate colorless products. This reaction occurs with little or no physical quenching. The solutions contained dyes in low concentrations and had optical densities ranging from 0.12 to 016 to minimize the possibility of $^1O_2$ quenching by the dyes. The photooxidation of DPBF was monitored from 20 s to 200 s. The $^1O_2$ quantum yield was calculated relative to optically matched solutions and comparing the quantum yield of DPBF photooxidation after sensitization by the compound of interest to that of Rose Bengal (RB) ($\phi[^1O_2]$ 0.76 in DMSO) as a reference compound according to Equation (vii).

$$\phi\Delta_c = \phi\Delta_{RB} \times m_c/m_{RB} \times F_{RB}/F_c \dots\dots\dots\dots\dots\dots\dots\text{ (vii)}$$

where c denotes a complex, and RB denotes Rose Bengal. $\phi\Delta$ is the $^1O_2$ quantum yield, and m is the slope of the plot of DPBF absorbance at 417 nm vs. irradiation time. F is the absorption correction factor, which is given by Equation (viii).

$$F = 1-10^{OD} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\text{ (viii)}$$

Where, OD is the optical density at the irradiation wavelength.

Intracellular ROS Generation[10]

The intracellular ROS generation was studied by fluorescence assisted cell sorting using 2',7'-dichlorofluorescin diacetate (DCFDA) assay. In the typical assay, the HeLa cells are pre-treated with complex **RuL4** (10 μM) for 4 h, followed by photoirradiation with red light (600−720 nm, 30 J/cm^2) for an hour and then treated with DCFDA; finally, they are analyzed by EVOS M5000 fluorescent live cell imager (Thermo Fisher Scientific, USA).

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