Electronic Supplementary Information for

Antitumor and antitrypanosomal activities of a new class of trinuclear ruthenium complexes with orthometalated phenazine ligands

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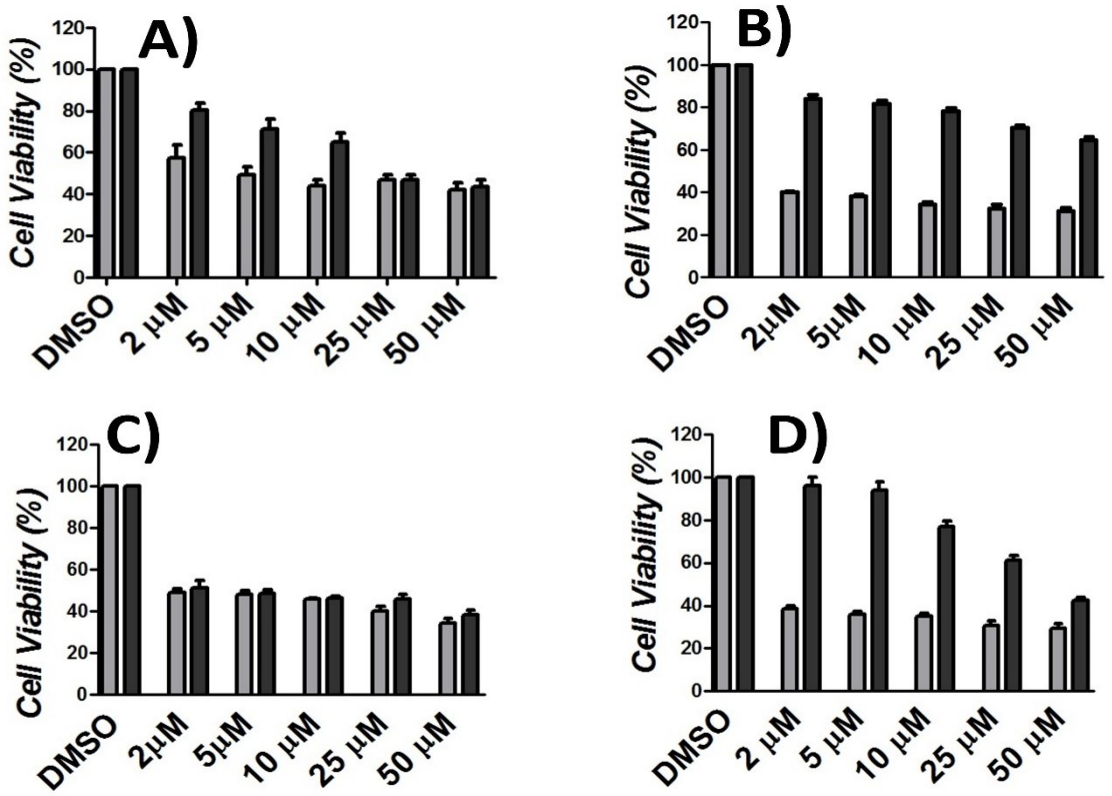

Figure ESI-1. Cytotoxic effect in murine melanoma (B16F10 cells) for complexes **1**-**4** (**A**-**D**) (light gray bars) and their free ligands (dark gray bars) in a concentration range of 2-50 µM after 24h treatment. Data is shown as mean ± standard error of mean (S.E.M.) of three independent experiments. Results did not show statistically significant differences to control experiments, considering $p < 0.05$ according to ANOVA and Newman-Keuls.

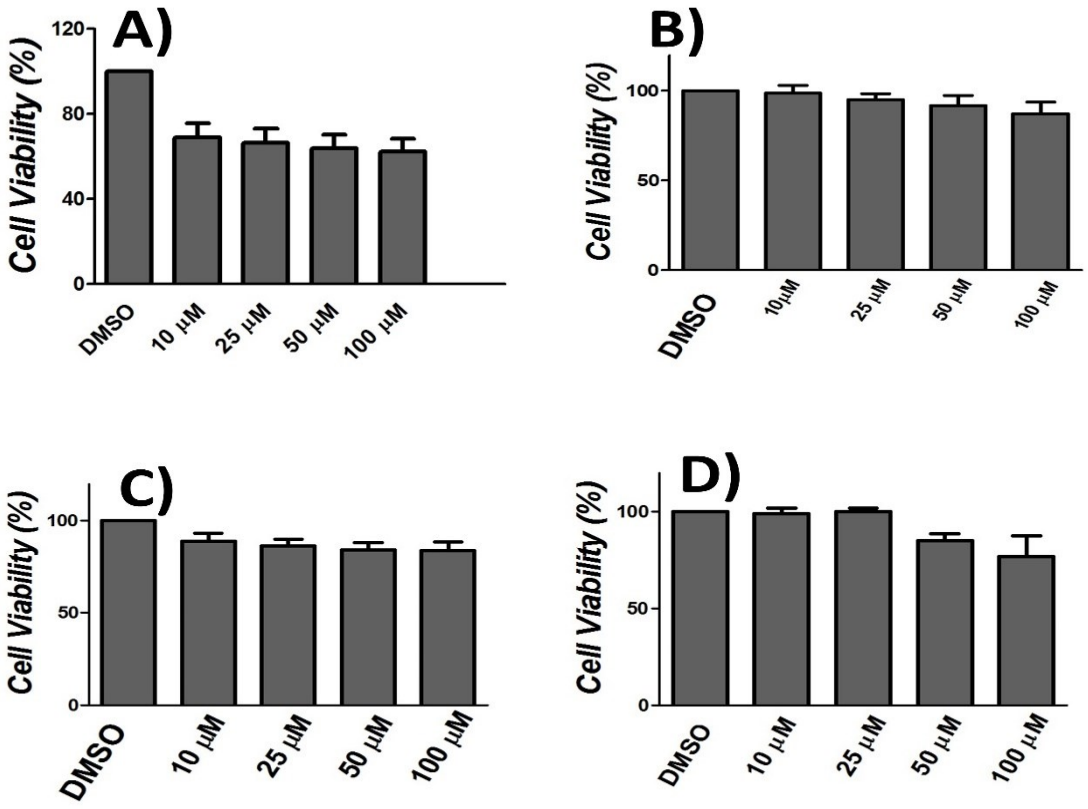

Figure ESI-2. Cytotoxic effect in non-cancer L929 cells for complexes **1**-**4** (**A**-**D**) in a concentration range of 10-100 µM after 24h treatment. Data is shown as mean ± standard error of mean (S.E.M.) of three independent experiments. Results did not show statistically significant differences to control experiments, considering $p < 0.05$ according to ANOVA and Newman-Keuls.

Complexes stability assay in different culture media and DMSO solution

Stability tests were performed by monitoring the complexes' absorption spectra at Agilent spectrophotometer (UV-Visible (8453/8454). The stock solution of complexes **1**-**4** (for anticancer assays) and **2**-**5** (for trypanocidal assays) were prepared in DMSO to a given concentration of 10^{-3} M. The assay tests were carried out in three different solutions: RPMI medium (Roswell Parking Memorial Institute – Gibco), RPMI-1640 medium (Roswell Park Memorial Institute - Sigma-Aldrich) and DMSO solution. Then, an aliquot of complexes solution was taken and transferred to a 1 mL of solution (RPMI, RPMI-1640 and DMSO) in a cuvette to keep complexes' final concentration at 10^{-5} M. The spectra were recorded as a function of time in 24 h, 48 h and 72 hours. The data analyses were performed at Origin® 8.6 software.

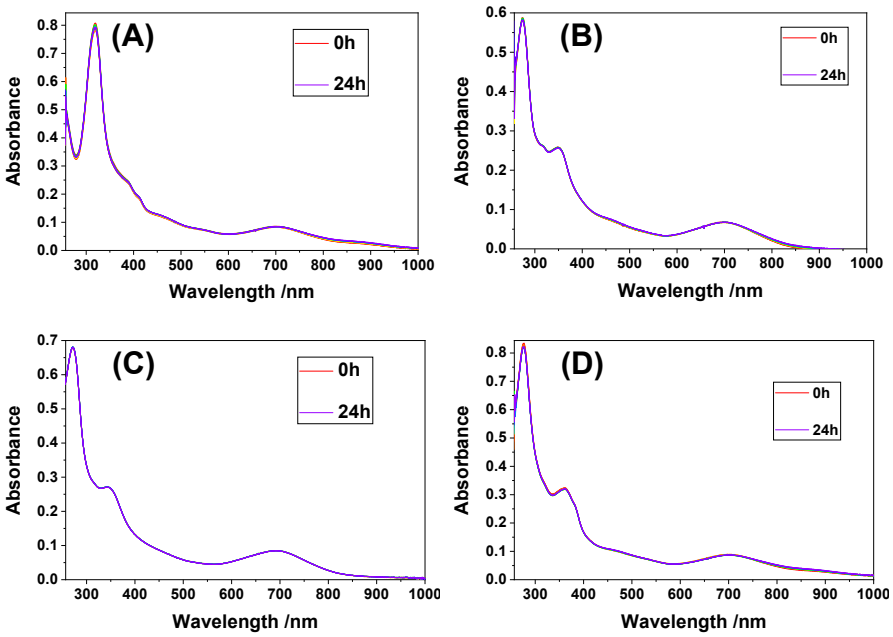

Figure ESI-3. Stability of complexes **1**-**4** (**A**-**D**) in the culture medium used for B16F10 and L929 cell lines cytotoxicity assays.

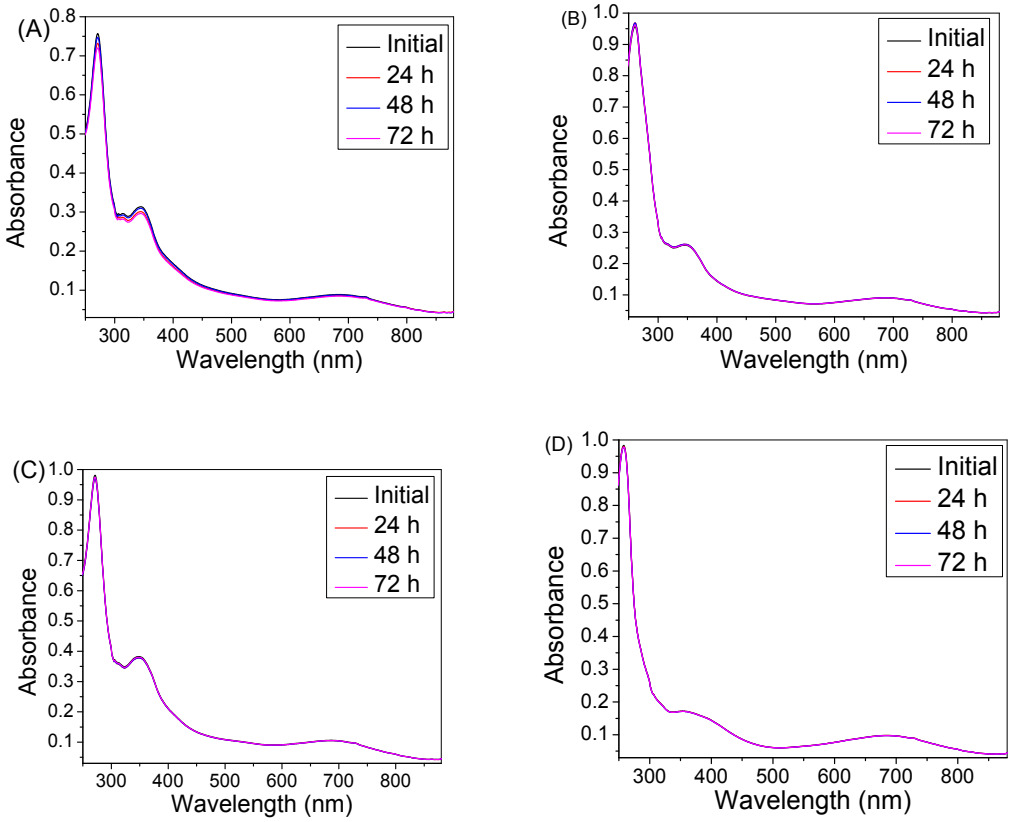

Figure ESI-4. Stability of complexes **2**-**5** (**A**-**D**) in the culture medium (RPMI – 1640) used for *Trypanosoma cruzi* assays.

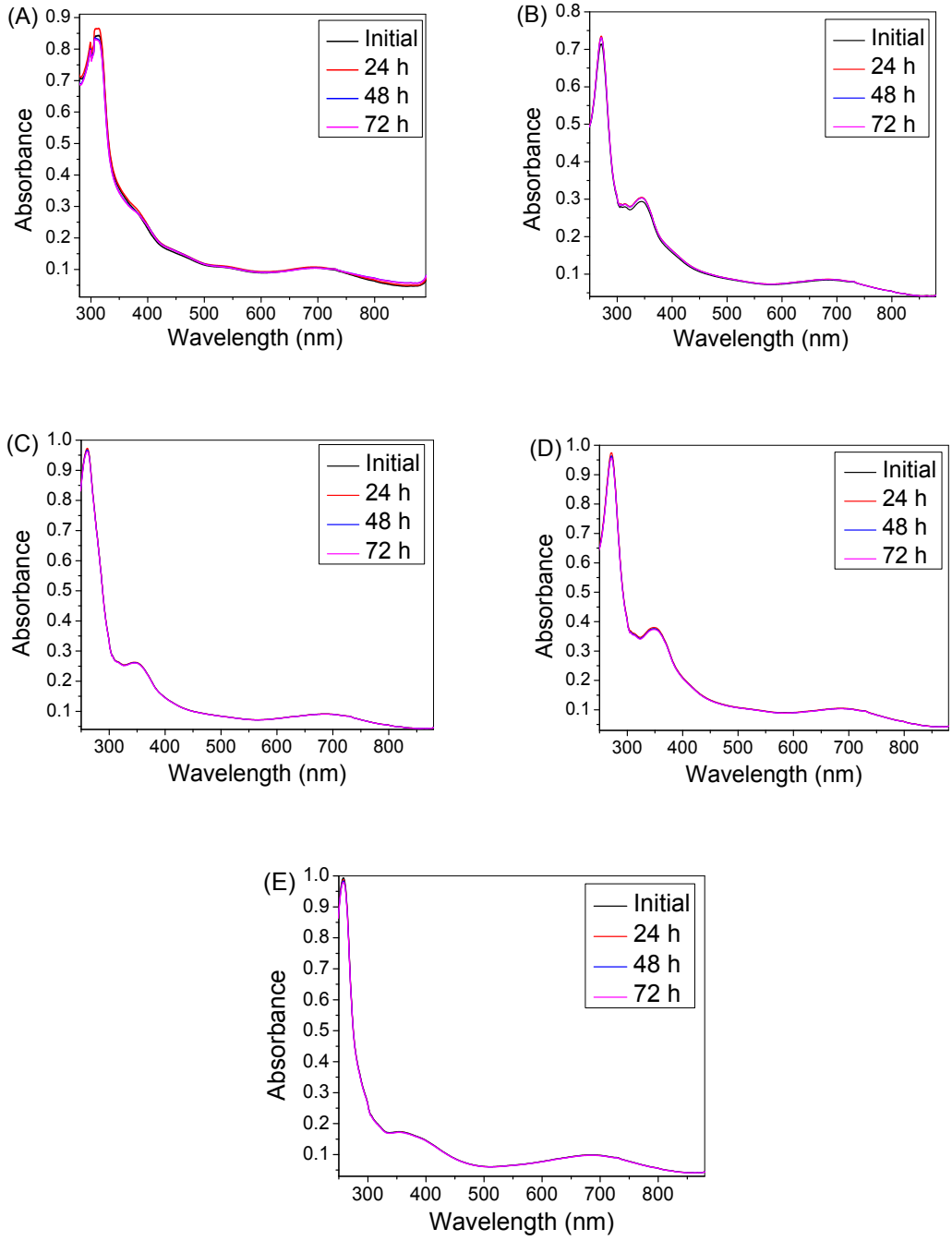

Figure ESI-5. Stability of complexes **1**-**5** (**A**-**E**) in DMSO solution used to prepared the stock solutions of complexes to perform biological assays.

Comments on cell incubation medium: In vitro biological activities of complexes and ligands were assessed by standard procedures described in literature.[i] In both experiments (antitumor and trypanocidal assays) the specific cells were incubated with compounds in the presence of cell culture medium. The RPMI medium is used to

maintain the cells' activities. Cells and compounds were incubated with RPMI medium, and then supplemented, bearing the final compositions described in Table **1**.

Table 1. Differences in final composition of cell culture media used in anti-Trypanosoma and cancer cells cytotoxicity assays, after supplementation.

Cell culture medium - anti-Trypanosoma activity	**Cell culture medium – cancer cells cytotoxicity**
RPMI 1640 – Sigma-Aldrich Without phenol red, sodium bicarbonate and sodium pyruvate With L-Glutamine	RPMI 1640 – Gibco Without phenol red, sodium bicarbonate and sodium pyruvate With L-Glutamine and 25 mM of HEPES
2.0 g L^{-1} of Sodium Bicarbonate	1.5 g L^{-1} of Sodium Bicarbonate
2.8 g of HEPES (11 mM)	-
2.5 mL of penicillin/streptomycin (Pen Strep, 10,000 U/mL, Gibco)	1% (v/v) of penicillin/streptomycin (10,000 U/mL, Gibco)
10% (v/v) of Fetal Bovine Serum (Gibco)	5% (v/v) of Fetal Bovine Serum (Gibco)
-	4.5 g of glucose

As shown in Table 1, the culture media from different sources have slightly different composition (different concentration of HEPES, fetal bovine serum and presence of glucose). Also, these media were supplemented differently in each biological assay, due to specific cell growth needs. These differences in final compositions are responsible for the different solubilities observed for compound **1**, especially regarding the ionic strength in cell culture medium for antitrypanosomal activity, which is supplemented with a higher concentration of sodium bicarbonate than that used for cancer cells culture medium. Also, cell culture medium for antitrypanosomal activity used more Fetal Bovine Serum, as the culture is grown in 3 days, in comparison to cancer cells culture medium which grows for 2 days. Finally, the occurrence of precipitate in the biological assays were monitored with a FLoid™ Cell Imaging Station, ThermoFisher Scientific microscope.

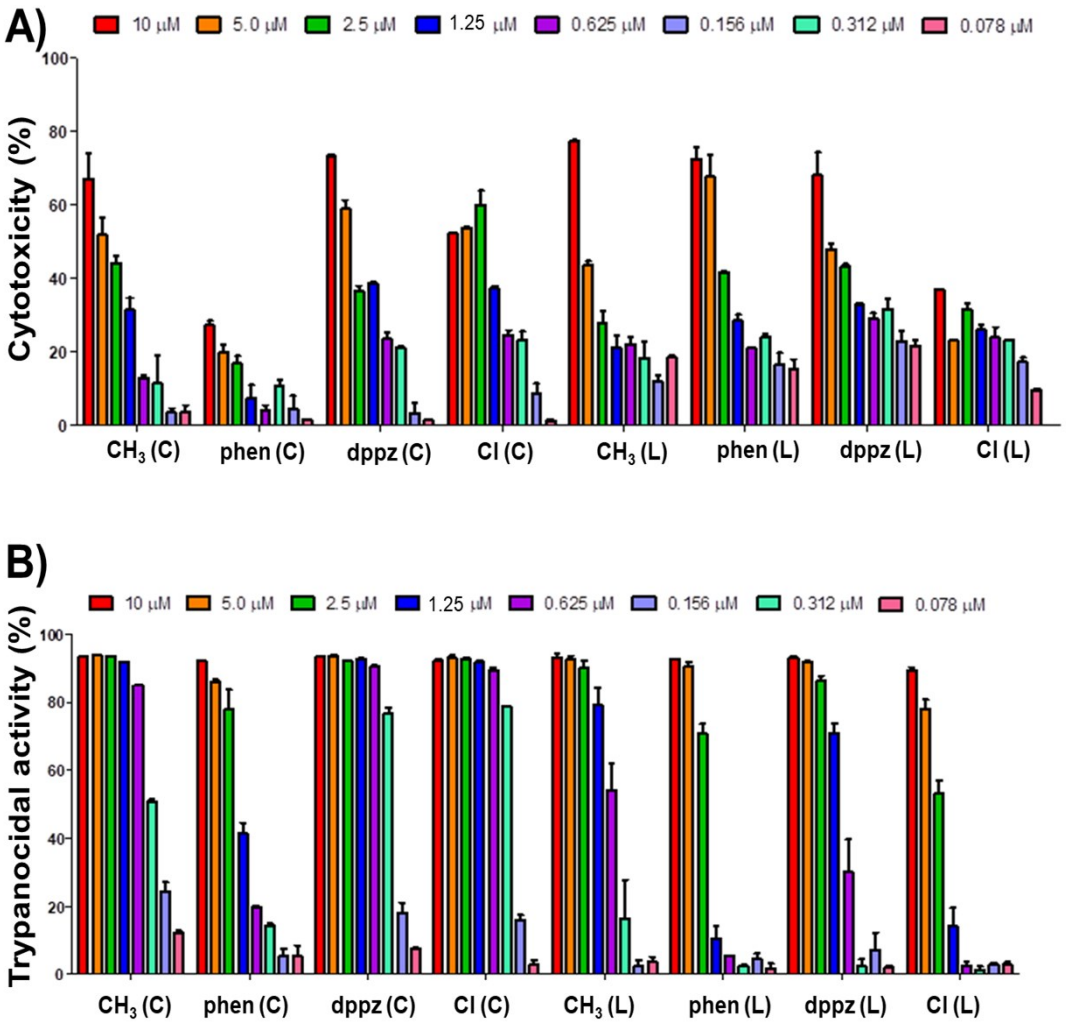

Figure ESI-6. (**A**) Cytotoxic effect in LLC-MK2 cell line for complexes **2**-**5** (CH_3 (C), phen (C), dppz (C) and Cl (C)) and their free ligands (CH_3 (L), phen (L), dppz (l) and Cl (L)) in a concentration range of 10 - 0.078 μM after 72h treatment. (**B**) Trypanocidal activity for *T. cruzi,* amastigotes form, for complexes **2**-**5** (CH_3 (C), phen (C), dppz (C) and Cl (C)) and their free ligands (CH_3 (L), phen (L), dppz (l) and Cl (L)) in a concentration range of 10 - 0.078 μM after 72h treatment. One replicate out of three is shown. C = complexes; L = ligands.

Table 2. Inhibition of Trypanosoma cruzi (amastigote forms and epimastigote forms) and cytotoxicity for LLC-MK2 monkey kidney cells for complexes **2**-**5** and their free ligands.

	LLC-MK2 /µM			**Mean ± SD**	**Amastigotes / µM**			**Mean ± SD**	**SI CC_{50}/ IC_{50}**	**Epimastigote /µM**			**Mean ± SD**
Complex **2**	1.42	3.17	2.74	2.44 ± 0.91	0.282	0.230	0.235	0.249 ± 0.03	9.80	1.36	1.06	1.16	1.19 ± 0.13
Complex **3**	0.943	3.98	1.75	2.22 ± 1.57	0.305	0.288	0.241	0.278 ± 0.03	8.00	1.80	1.83	2.58	2.07 ± 0.36
Complex **4**	0.454	3.62	1.66	1.91 ± 1.60	0.225	0.231	0.174	0.210 ± 0.03	9.11	1.38	1.31	2.33	1.67 ± 0.46
Complex **5**	7.23	>10	> 10	9.08 ± 1.60	2.23	1.39	1.45	1.69 ± 0.47	4.28	1.51	2.30	4.14	2.65 ± 1.10
Dppz	6.50	3.94	4.41	4.95 ± 1.37	0.573	0.867	0.509	0.650 ± 0.19	7.62	1.16	6.01	4.89	4.02 ± 2.07
DppzCH_3	5.93	4.71	4.82	5.15 ± 0.67	0.663	0.593	0.561	0.606 ± 0.05	8.51	1.06	2.98	3.91	2.65 ± 1.19
DppzCl	>10	>10	> 10	> 10	1.75	2.50	1.20	1.81 ± 0.65	> 5.52	0.531	0.581	0.651	0.59 ± 0.05
Phen	4.64	2.84	4.39	3.96 ± 0.97	1.88	1.96	1.46	1.76 ± 0.27	2.24	4.76	9.49	7.77	7.34 ± 1.95
BZ	>100	>100	> 100	> 100	6.55	4.98	5.71	5.74 ± 0.78	> 17.4	20.2	21.4	22.5	21.4 ± 0.94

Concentration was shown in micromolar values. IC_{50}: inhibitory concentration for 50% of amastigote forms (Tulahuen strain); CC_{50}: cytotoxic concentration for 50% of mammalian cells; SI: selectivity index, given by the ratio CC_{50}/IC_{50}; BZ: Benznidazole; SD: Standard Deviation. IC_{50}: inhibitory concentration for 50% of epimastigote forms (Tulahuen strain); BZ: Benznidazole; SD: Standard Deviation

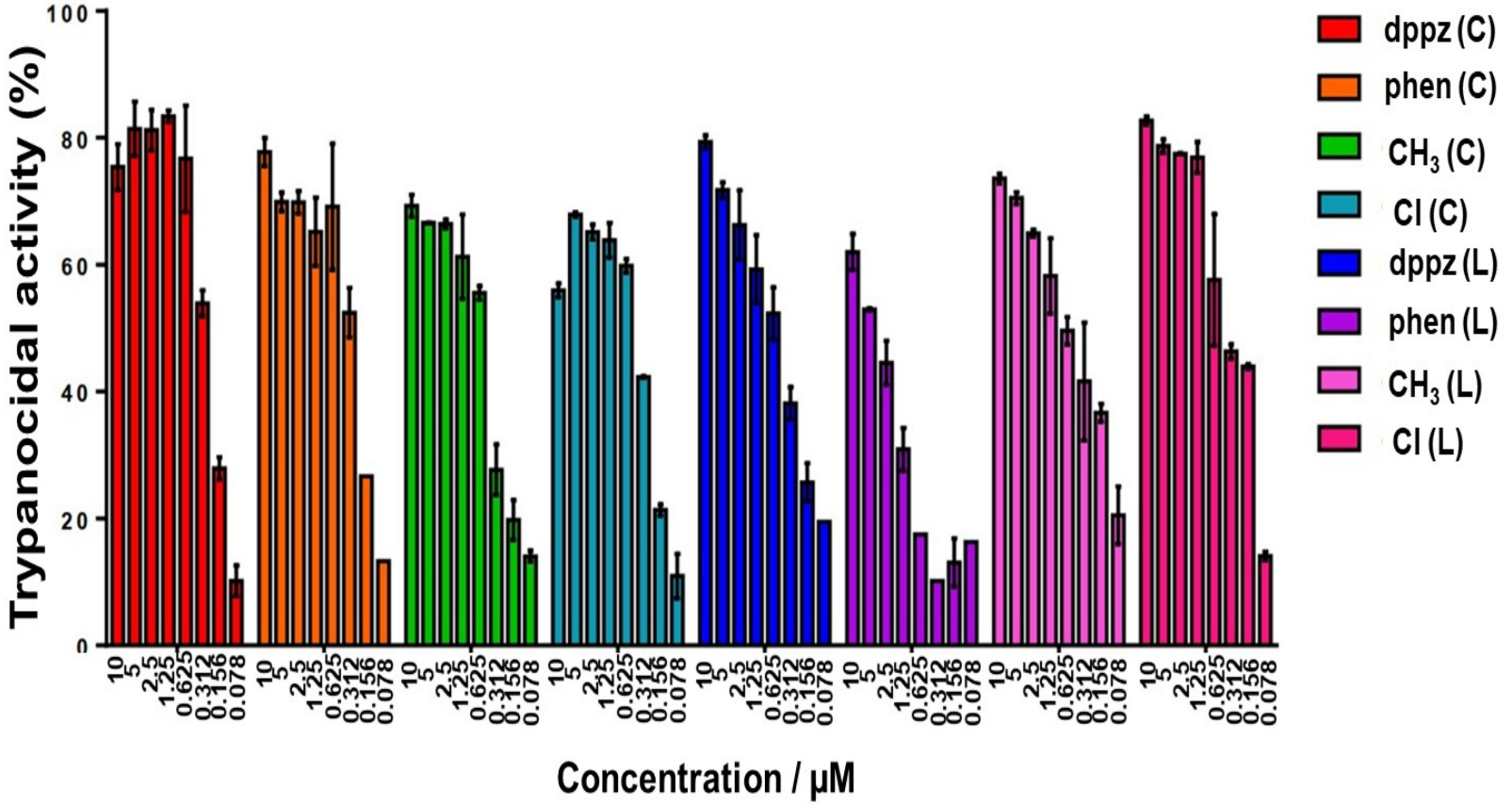

Figure ESI-7. Trypanocidal activity, epimastigote forms for complexes **2**-**5** (dppz (C), phen (C), CH_3 (C) and Cl (C)) and their free ligands (dppz (L), phen (L), CH_3 (l) and Cl (L)) in a concentration range of 10 - 0.078 µM after 72h treatment. One replicate out of three is shown. C = complexes; L = ligands.

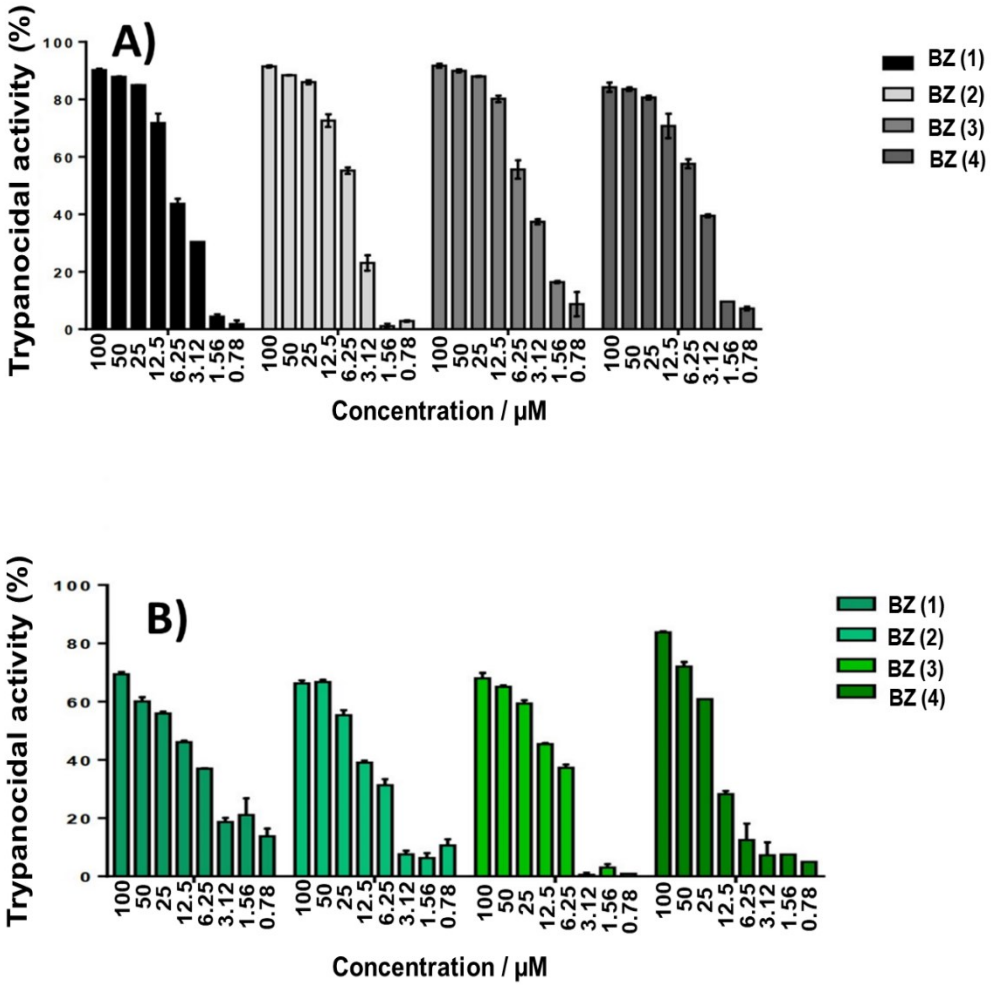

Figure ESI-8. Trypanocidal activity for Benznidazole (BZ) in amastigote (**A**) and epimastigote (**B**) forms of *T. cruzi* in a concentration range of 100 - 0.78 µM after 72h treatment. Four replicates (BZ (1), BZ (2), BZ (3) and BZ (4)) are shown.

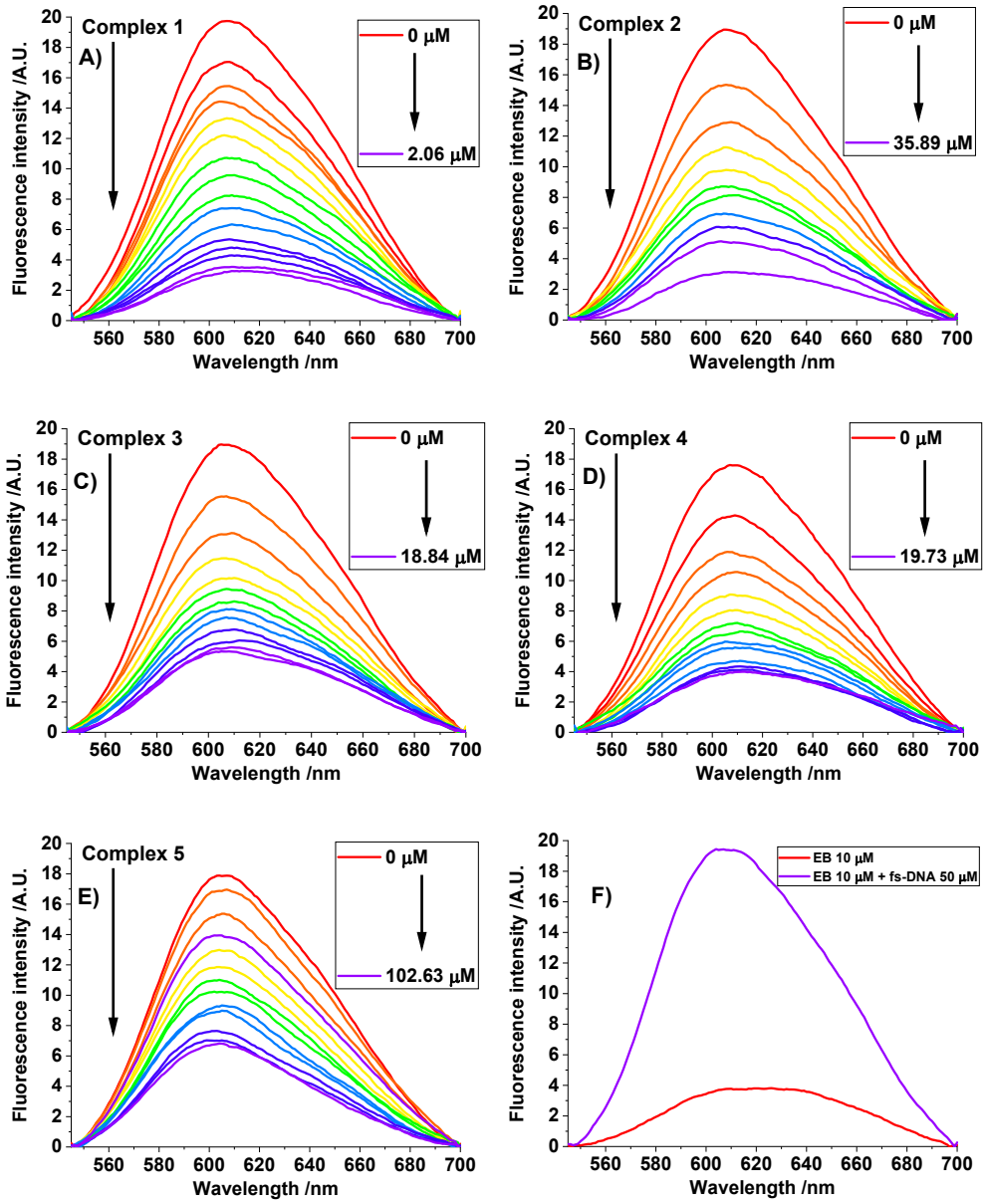

Figure ESI-9. Interaction of complexes with DNA through ethidium bromide displacement assay: fluorescence spectra for complexes **1**-**5 (A-E)** and fluorescence spectra of EB in the presence and absence of *fs*-DNA (F).

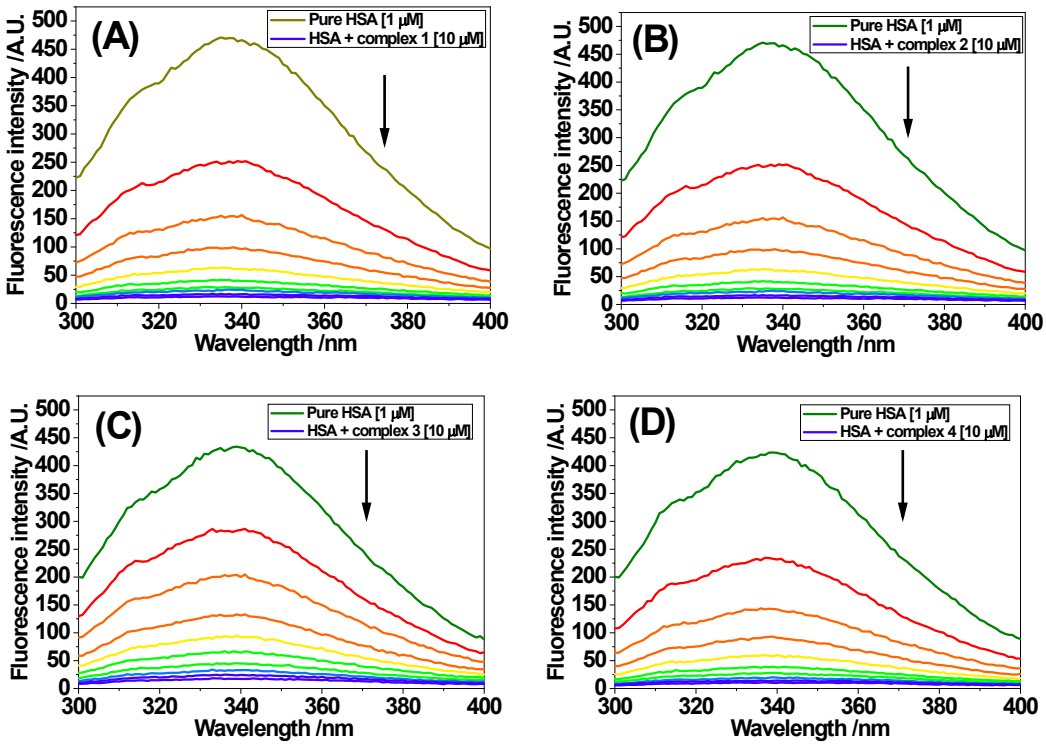

Figure ESI-10. Fluorescence spectra of HSA as a function of concentration of complexes **1**-**4** (**A**-**D**).

Details of the Procedure: interaction of complexes 1-4 with HSA and Stern-Volmer mathematical model

The titration data was treated using the modified Stern-Volmer model, equation 1:

$$K_{ap} = \left[\frac{F_0}{F} - 1\right]\frac{1}{[S]} = (K_D + K_S) + K_D.K_S[S] \qquad \text{Eq 1}$$

Where:

K_{ap} = apparent quenching constant;

K_D = dynamic fluorescence quenching constant;

K_S = static fluorescence quenching constant.

The graph of K_{ap} vs [**1**] produces a linear tendency which intersects the y-axis at the point where $K_D + K_S$ is obtained, and the slope is related to the product of the constants $K_D.K_S$. To determine the bimolecular quenching rate constant (k_q), Equation 2 was used, where t_0 is the mean life time of the biomolecules in the absence of the suppressor, (10^{-8} s)[2,3]

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$$K_{ap} = k_q \times t_0 \qquad \text{Eq 2}$$

Florescence quenching data was also analyzed to obtain binding parameters (K_b) for the interaction of complex and HSA and the number of binding sites (n) were calculated using Equation 3.

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_b + n\, log[Q] \qquad \text{Eq 3}$$

The thermodynamic parameters for HSA were calculated at three temperatures (298 K, 304 K and 310 K) for complex **1**-**5** using the Van't Hoff Equation (Equation 4) [2].

$$\ln Kb = \frac{\Delta G}{RT} + \frac{\Delta S}{R} \qquad \text{Eq 4}$$

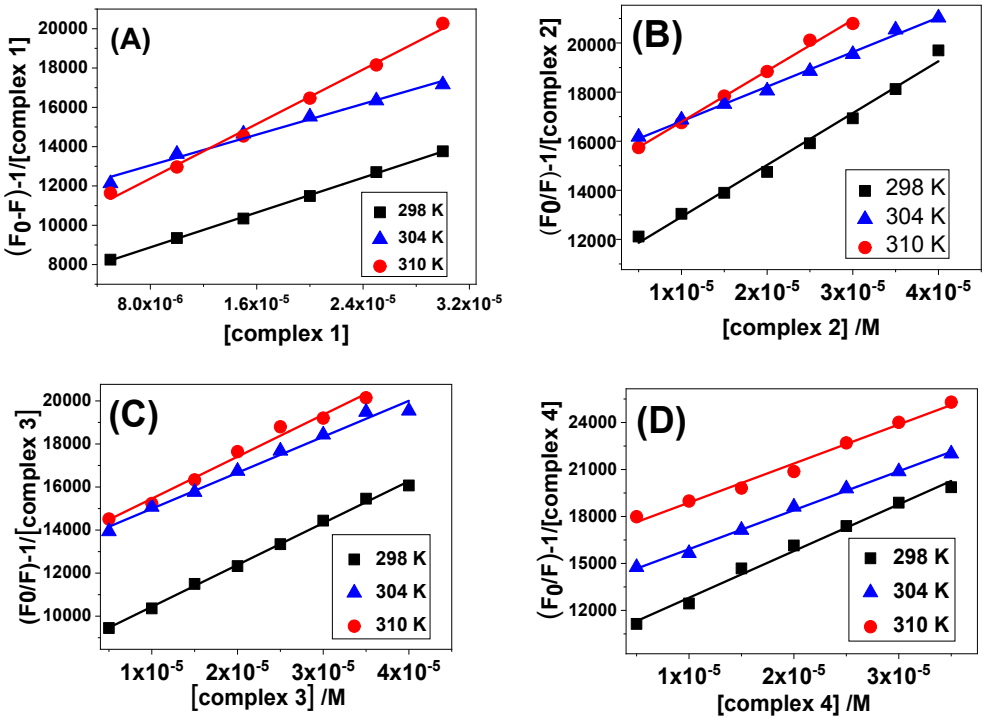

Figure ESI-11. Stern-Volmer ((F_0/F) 1- [complex]) curves for suppression of HSA fluorescence (λ_{exc} = 280 nm) for different concentrations of the complexes **1**-**4** (**A**-**D**), at different incubation temperatures (298 K, 304 K and 310 K).

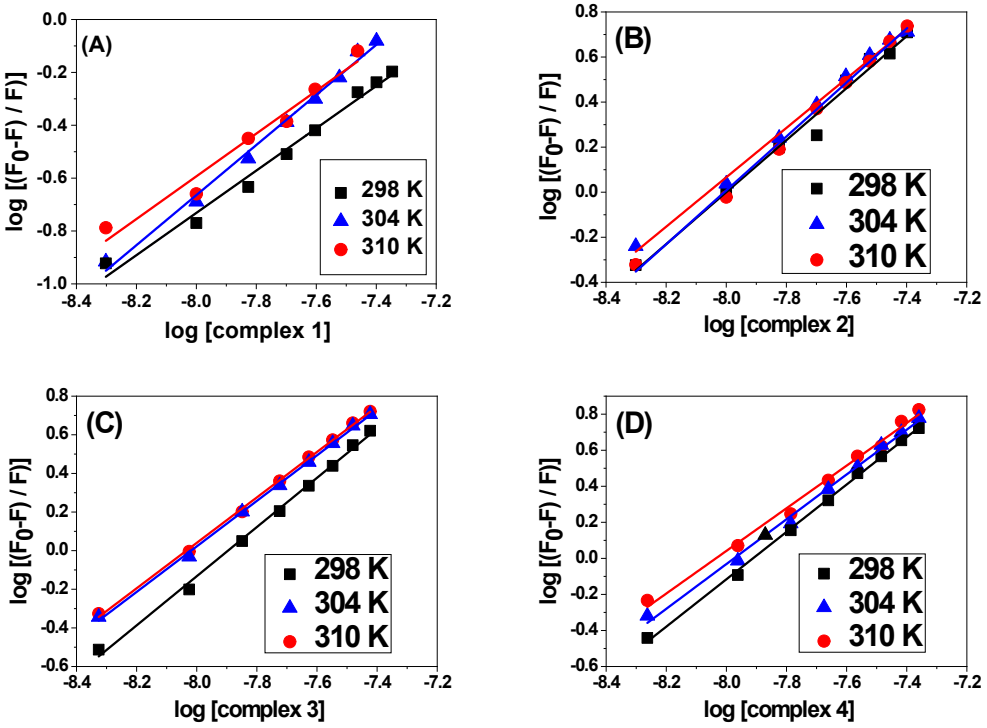

Figure ESI-12. Graph to find the binding constant (K_b) and the number of suppressor binding sites (n) at different concentrations of the complexes **1**-**4** (**A**-**D**) at different incubation temperatures (298 K, 304 K and 310 K).

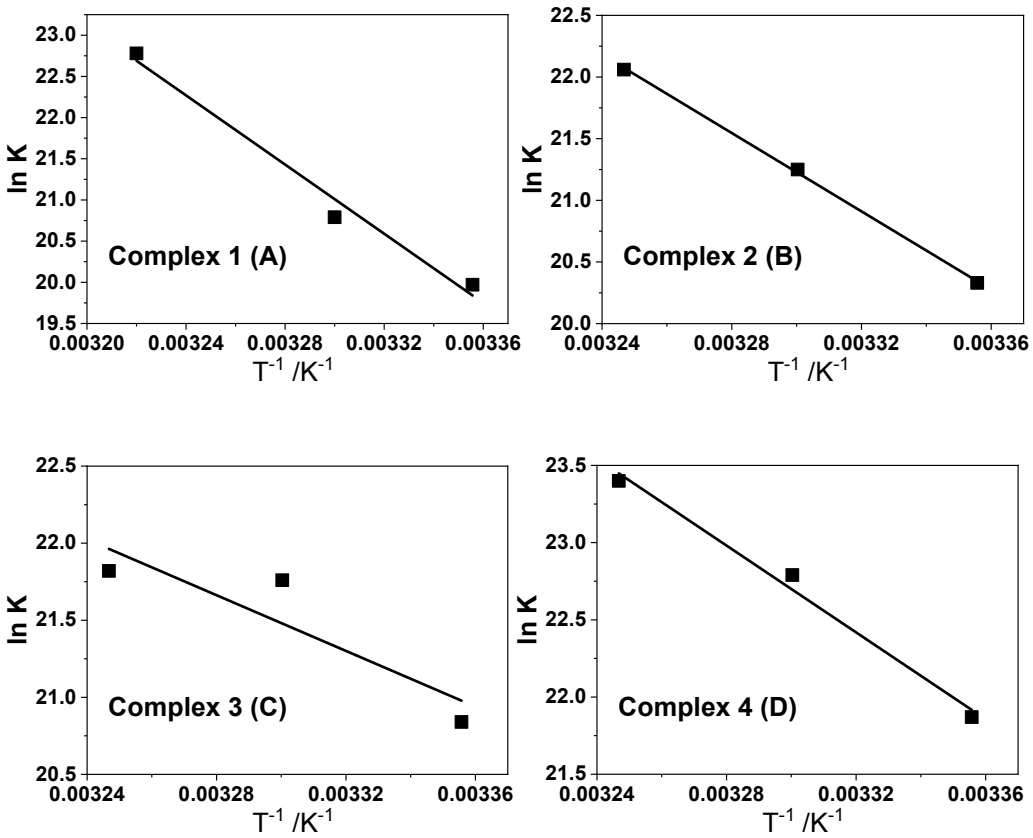

Figure ESI-13. Van't Hoff, HSA (10^{-6} M) graphs of the complexes **1**-**4** (**A**-**D**) at different incubation temperatures (298 K, 304 K and 310 K).

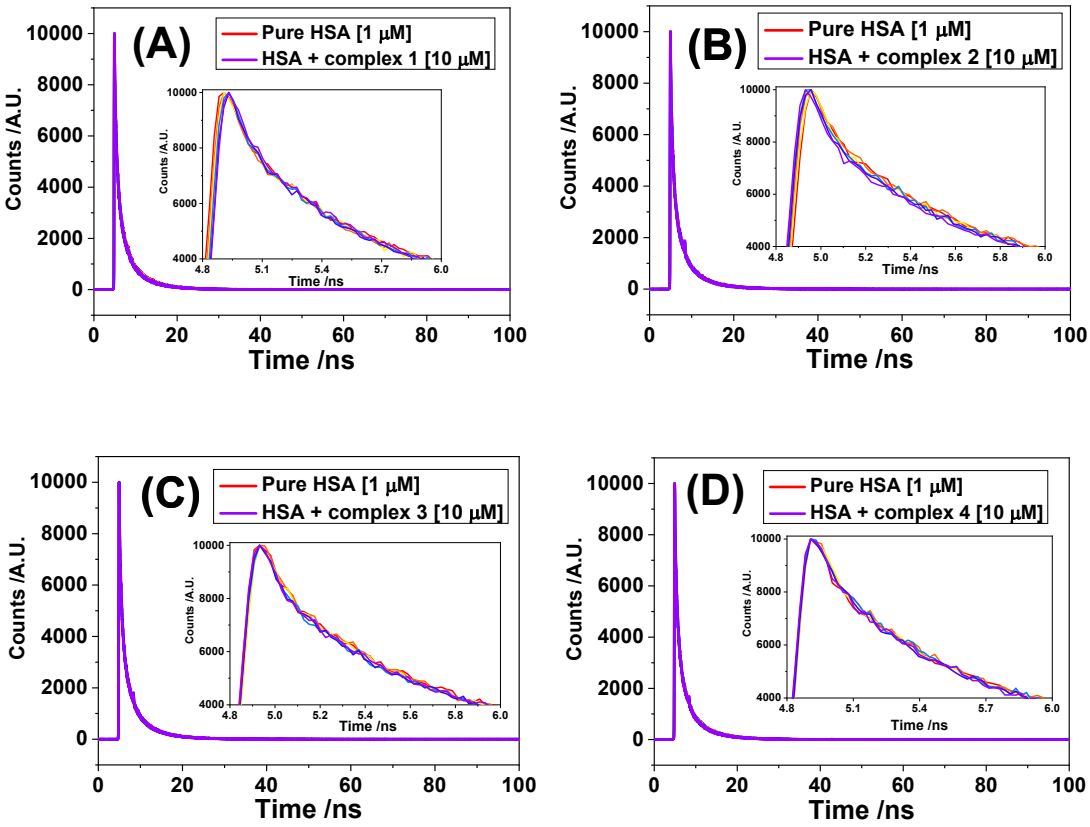

Figure ESI-14. Fluorescence decay profile of the interaction between complexes (**1**-**4**, **A**-**D**) and HSA, in tris buffer (pH 7.2), λ_{exc} 295 nm, at 25 °C.

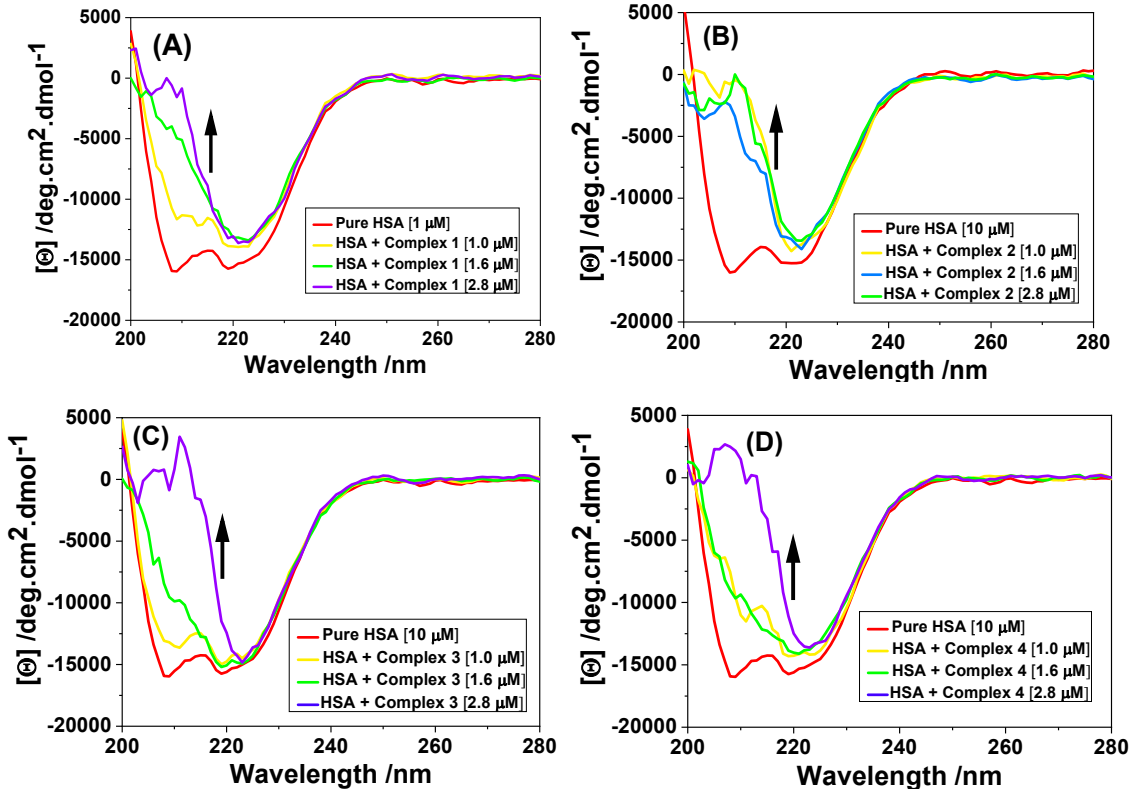

Figure ESI-15. HSA CD absorption spectra [1.0 μM] in the presence of the complexes (**1**-**4**, **A**-**D**) in Tris-HCl Buffer pH 7.4 at room temperature.

Table 3: Viscosity data for EtBr

[EtBr]/ [DNA]	t_1	t_2	t_3	Time Average (s)	(t – t0)	$(\eta/\eta_0)^{1/3}$
Buffer only	235	235	235	235 t_0	---------	----------
Buffer + DNA	300.8	300.8	300.9	300.8	65.8 η_0	1
0.05	324	330	332	329	94	1.42
0.10	332	330	331	331	96	1.46
0.15	347	337	340	341	106	1.61
0.20	346	357	352	352	117	1.76
0.25	354	356	355	355	120	1.83

Table 4: Viscosity data for complex **1**

[dppn]/ [DNA]	t_1	t_2	t_3	Time Average (s)	(t – t0)	$(\eta/\eta_0)^{1/3}$
Buffer only	235	235	235	235 t_0	---------	----------
Buffer + DNA	300.8	300.8	300.9	300.8	65.8 η_0	1
0.05	320	319	319	319	84.2	1.28
0.10	318	320	322	320	84.9	1.29
0.15	325	325	324	324	89	1.36
0.20	333	332	330	331	96.7	1.47
0.25	336	334	335	335	100.01	1.52

Table 5: Viscosity data for complex **2**

[dppz]/ [DNA]	t_1	t_2	t_3	Time Average (s)	(t – t0)	$(\eta/\eta_0)^{1/3}$
Buffer only	239	239	239	239 t_0	---------	----------
Buffer + DNA	330	330	330	330	91 η_0	1

0.05	337	336	337	337	98.3	1.08
0.10	347	346	345	346	107.3	1.18
0.15	343	344	343	344	105.6	1.16
0.20	350	353	352	352	113.7	1.25
0.25	348	352	351	350	111.9	1.23

Table 6: Viscosity data for complex **3**

[dppzCH$_3$]/ [DNA]	**t_1**	**t_2**	**t_3**	**Time Average (s)**	**(t – t0)**	**$(\eta/\eta_0)^{1/3}$**
Buffer only	237	237	235	237 **t_0**	---------	----------
Buffer + DNA	312	312	311	312	75 η_0	1
0.05	318	318	320	319	82.5	1.10
0.10	326	324	324	325	88.5	1.18
0.15	330	326	328	327	90	1.20
0.20	329	330	332	329	92.2	1.23
0.25	329	330	330	330	93.7	1.25

Table 7: Viscosity data for complex **4**

[dppzCl/ [DNA]	**t_1**	**t_2**	**t_3**	**Time Average (s)**	**(t – t0)**	**$(\eta/\eta_0)^{1/3}$**
Buffer only	236	236	237	236 **t_0**	---------	----------
Buffer + DNA	310	310	310	310	74 η_0	1
0.05	313	315	313	314	78.4	1.06
0.10	320	319	319	319	82.8	1.12
0.15	323	323	325	324	88.06	1.19
0.20	321	323	324	323	87.32	1.18
0.25	322	324	322	322	85.8	1.16

Table 8: Viscosity data for complex $[Ru(bpy)_3]^{2+}$

$[Ru(bpy)_3]^{2+}$/ [DNA]	t_1	t_2	t_3	Time Average (s)	$(t - t_0)$	$(\eta/\eta_0)^{1/3}$
Buffer only	239	239	239	239 t_0	---------	----------
Buffer + DNA	330	330	330	330	91 η_0	1
0.05	327	328	328	328	89.2	0.98
0.10	324	327	326	326	87.4	0.96
0.15	330	333	333	333	94.6	1.04
0.20	329	328	329	329	90.1	0.99
0.25	333	333	331	333	97.7	1.03

[i] Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, *65*(1-2), 55-63.

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