Analysis of Antiproliferative activity of New Half-sandwich Arene Ru(II) thiophene based aroylhydrazone complexes

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Materials, Methods and Crystal data collection

Commercially available $RuCl_3.3H_2O$ from LobaChemie was used. 2-thiophenaldehyde and derivatives of heterocyclic hydrazides were purchased from Sigma Aldrich. The starting materials $[(\eta^6\text{-}p\text{-cymene})RuCl_2]_2$ were prepared by literature methods.[1,2] Chemically pure and analar grade reagents were used for all the reactions. All the reagents used were chemically pure and analar grade. The solvents were freshly distilled before use by following standard procedures.[3] Boeties micro heating table was used to record the melting points and are uncorrected. The IR spectra of ligands and complexes were recorded within the range of 4000-400 cm^{-1} using Perkin-Elmer 597 spectrophotometer. A Cary 300 Bio UV–vis Varian spectrophotometer was utilized to record the electronic spectra of complexes in the range 800-200 nm. The ^{1}H-NMR spectra were recorded with Bruker 400 MHz instrument using TMS as internal reference in $CDCl_3$ and DMSO-d_6. A Micro mass thermo-scientific LTQ XL mass spectrometer was used for High-Resolution Mass Spectrometry of the complexes. Single crystals of complex **2** were grown by slow evaporation of a dichloromethane and petroleum ether solution at room temperature. The XRD data was acquired using Bruker D8 Quest with a Photon detector at 298 K temperature with graphite-monochromated MoK radiation (λ=0.71073Å). APEX 3 software was used to collect data as well as index reflections and unit cell properties. Reflections were categorised and processed, and files were scaled and corrected for absorption and structure determination using APEX 3 Software. The structure was solved using direct methods and then improved using the full-matrix least-squares calculation in SHELXL software.[4,5] Multi-scan absorption correction was applied. The cell parameters are a = 7.7107(12) Å, b = 19.424(3) Å, c = 14.081(2) Å, β = 96.242(5)°, volume = 2096.5(5) Å³. Space group: P 21/n. The final R values are R1 = 0.0349 and wR2 = 0.0972 for 5332 reflections with I > 2σ(I). All hydrogen atoms were geometrically fixed and collected to refine using a riding model. Complex **2** were drawn with ORTEP and the structural data have been deposited at the Cambridge Crystallographic Data Centre: CCDC for complex **2** is **2246503** respectively.

Experimental Procedures

Stability studies

The Time-dependent UV-visible spectral method has been used to examine the stability of the complexes. Complexes were dissolved in a minimum amount of 1% DMSO and then diluted with PBS buffer to 1×10^{-3} M concentration. The summaries of hydrolysis of

the complexes were monitored by electronic spectra over 72 h.[6]

Partition coefficients determination

The Hydrophobicity of the synthesized metal complexes **1-3** was determined to analyze the penetration behavior across the cell membrane. It is investigated in terms of partition coefficient (log P) using the "shake-flask" method between octanol/water phase partitions. Because of the substitution variation in different complexes, hydrophobicity differs. Octanol-saturated water (OSW) and water-saturated octanol (WSO) were prepared using analytical grade octanol (Sigma Aldrich) and doubly distilled water. Solubility and sample concentration play an important role in the measurements of given organic and aqueous medium compounds. Partition coefficients in a biphasic system were calculated by using the equation log P = log[(**1−3**)oct/(**1−3**)aq].[7] Complexes **1−3** (1 mg/mL; ethanol/water **1/3**) were diluted to 2, 4, 6, 8, and 10 μg/mL in water; alternatively, these (1 mg/mL) were diluted to 2, 4, 6, 8, and 10 μg/mL in octanol, respectively. Required quantities of the complexes (4 mg/mL) were shaken for one day at room temperature in equal volume (50/50). Once it achieved equilibrium, two phases were separated and centrifuged. As a final point, each phase drug concentration was measured by UV-visible spectroscopy.

Cell culture

A549 (lung carcinoma), HuH-7 (hepatocellular carcinoma), HeLa (cervical cancer), and non-cancerous NIH3T3 (human Fibroblast) cell lines were purchased from National Centre for Cell Science (NCCS), Pune. A549 cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (FBS; Gibco). HuH-7, HeLa and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). All of the media were supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL), and the cells were maintained in a humid atmosphere at 37 °C with 5% CO2.

***In vitro* cytotoxicity using an MTT assay**

The in vitro cytotoxicity of the complexes was measured by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The cells were plated in flat-bottomed 96-well plates (4 × 103 cells per well) and incubated at 37 °C for 24 h. The cells were added via serial dilution of Complexes 1-3 and cisplatin and then incubated at 37 °C for 48 h. At the end of the exposure, 30 μL MTT solution (5 mg/mL in PBS) was added to each

well. The MTT solution was removed from the wells after 4 h, and the purple MTT-formazan crystals were then dissolved by the addition of DMSO (100 μL). The absorbance in each well was measured at 490 nm using a microplate reader (Multiskan FC, ThermoScientific). DMSO blank assay has been performed before all cell assays.

Acridine orange-ethidium bromide (AO-EB) staining

4×10^3 of A549 cells were seeded in 24-well plates and incubated at 37 °C for 48 h. Complexes **1-3** (IC_{50} concentrations) were incubated with A549 cells. After incubation, AO (100 μg/mL) and EB (100 μg/mL) was added to each well (500 μL). After 5 min later, the cells were visualized via a fluorescence microscope (Olympus, BX-60, Japan), and the cell death were measured three random fields of the microscope.

Hoechst 33342 staining method

Hoechst 33342 staining was done using the method described earlier with slight modifications. 5×10^5 A549 cells were treated with IC_{50} concentration of the complexes **1-3** for 48 h in a 6-well culture plate and were fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. Cells were then stained with 50 μg mL-1 Hoechst 33342 for 30 min at room temperature. The cells undergoing apoptosis, represented by the morphological changes of apoptotic nuclei, were observed and imaged by epifluorescence microscope (Carl Zeiss, Germany).

Reactive Oxygen Species (ROS) Assay

For quantifying the intracellular ROS, 5×10^5 A549 cells were seeded on a 6-well plate containing cover slip and incubated overnight for attachment. After incubation, the cells were treated with fresh medium containing lethal dose of complexes **1-3** and incubated further for 48 h. At the end of incubation, cover slip was removed from the culture plate and stained with 40 μM of 2',7'-dichlorofluorescein-diacetate (DCFHDA) dye for 30 min. The stained cover slip was washed with PBS solution and visualized under fluorescence to record images.

Mitochondrial membrane potential(MMP)

The mitochondrial membrane potential of A549 was assessed using the fluorescent dye JC-1. A549 cells were cultured in a 6-well plate at a density of $1x10^5$ cells/well.

Following treatment, the culture medium was carefully removed and cells were washed twice with PBS. The cells were subsequently stained with 2 µM JC-1 for 20 min at 37˚C in the dark. Cells were analysed under a fluorescence microscope (20x, Floid cell imaging station, Life technologies,USA)

Annexin V-FITC/PI staining by flow cytometry method

A549 Cells have been seeded in a 6-well plate (10^5 cells/well)) and cultured at 37 °C for 48 hours. The cells have been treated with all the complexes (IC_{50} concentration) and incubated for 48 hours. Then, the cells were trypsinized, washed with PBS and stained with annexin V-FITC/PI according to the annexin V-FITC apoptosis detection kit. Finally, apoptosis induction has been assessed using a flow cytometer (SYSMEX, Japan), and the data have been analyzed by Flow Jo software. The cells that were not treated were employed as a control group.

UV-Vis spectra of complexes (1-3)

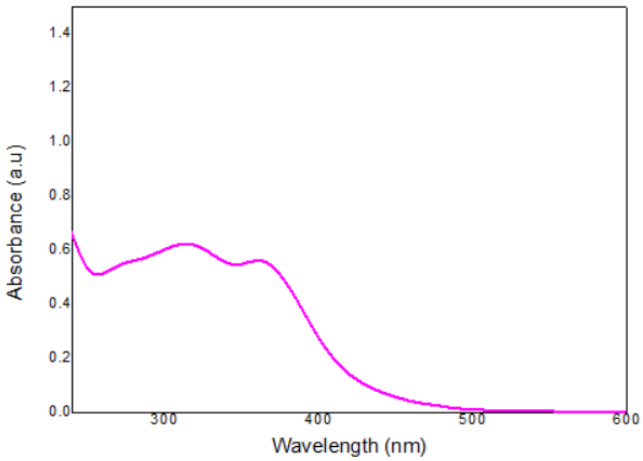

Figure S1. UV- Vis Spectrum of Complex **1** [A_{max} (nm): 363, 315, 270]

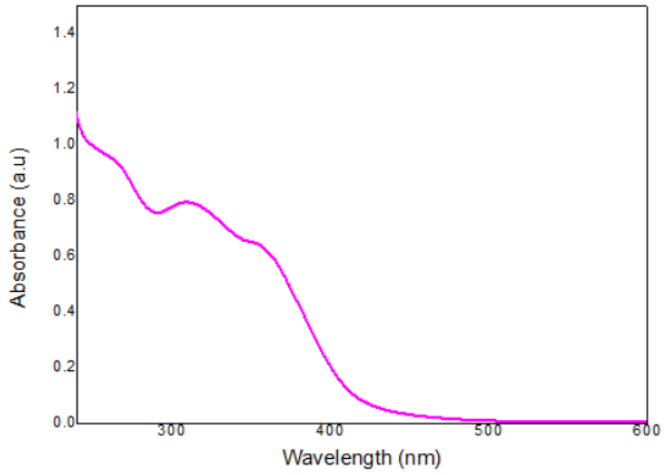

Figure S2. UV- Vis Spectrum of Complex **2** [A_{max} (nm): 356, 312, 265]

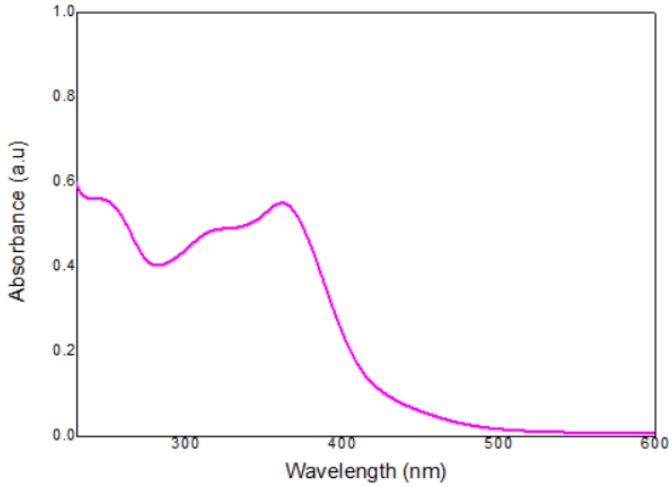

Figure S3. UV- Vis Spectrum of Complex **3** [A_{max} (nm): 360, 316, 250]

^{1}H and ^{13}C NMR spectra of the complexes(1-3)

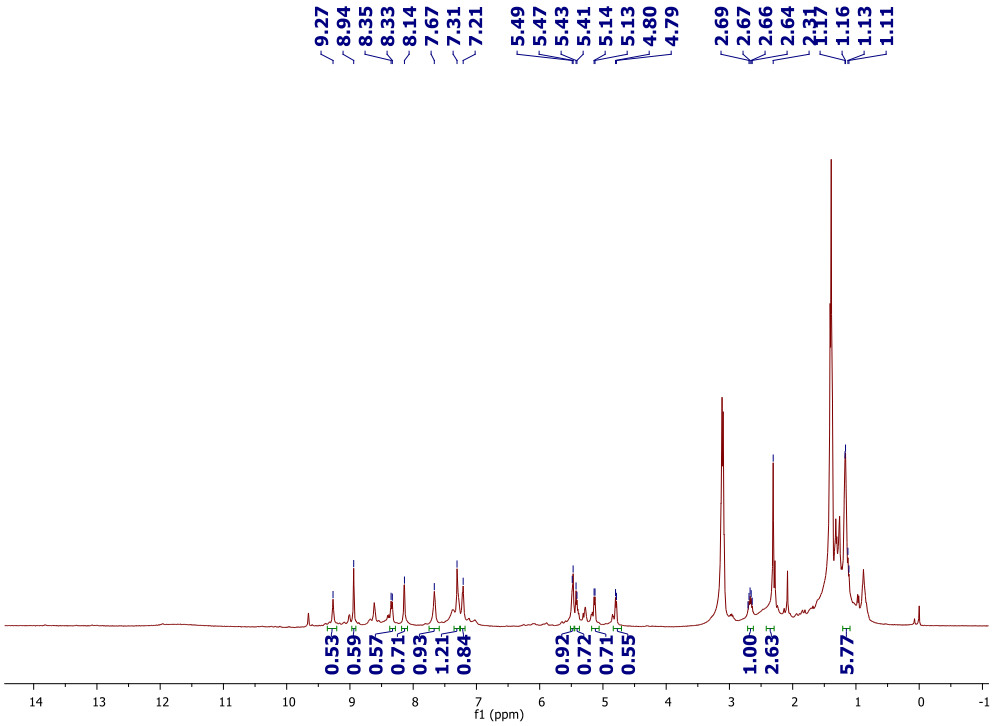

Figure S4. ^{1}H NMR spectrum of Complex **1**

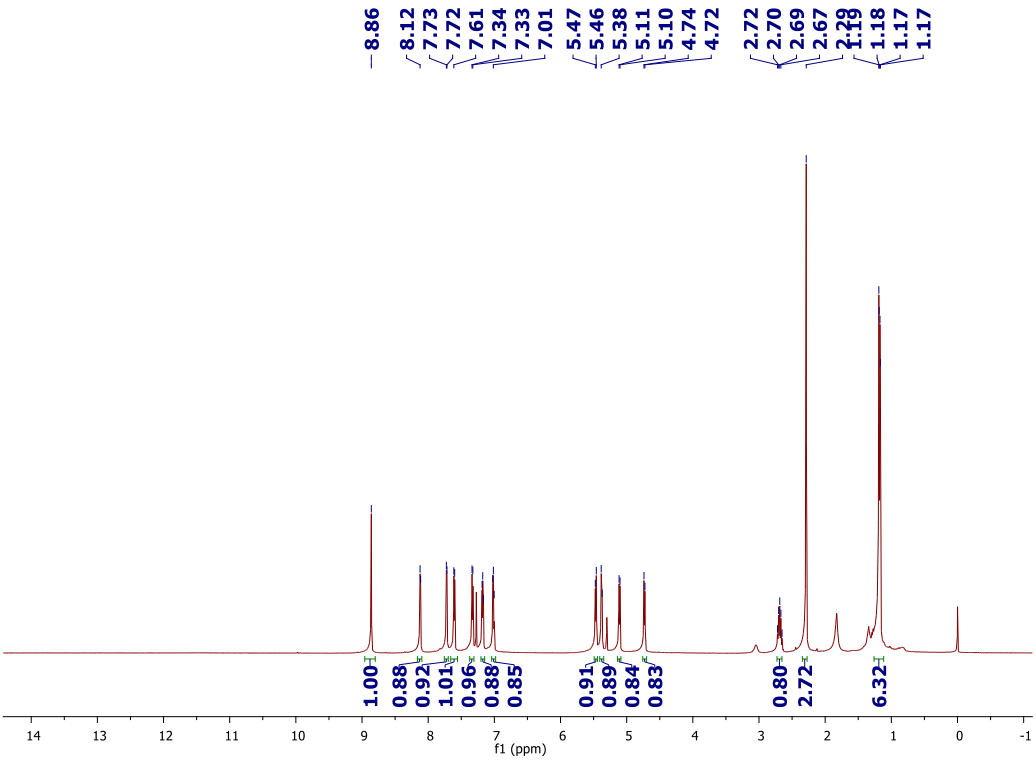

Figure S5. ^{1}H NMR spectrum of Complex **2**

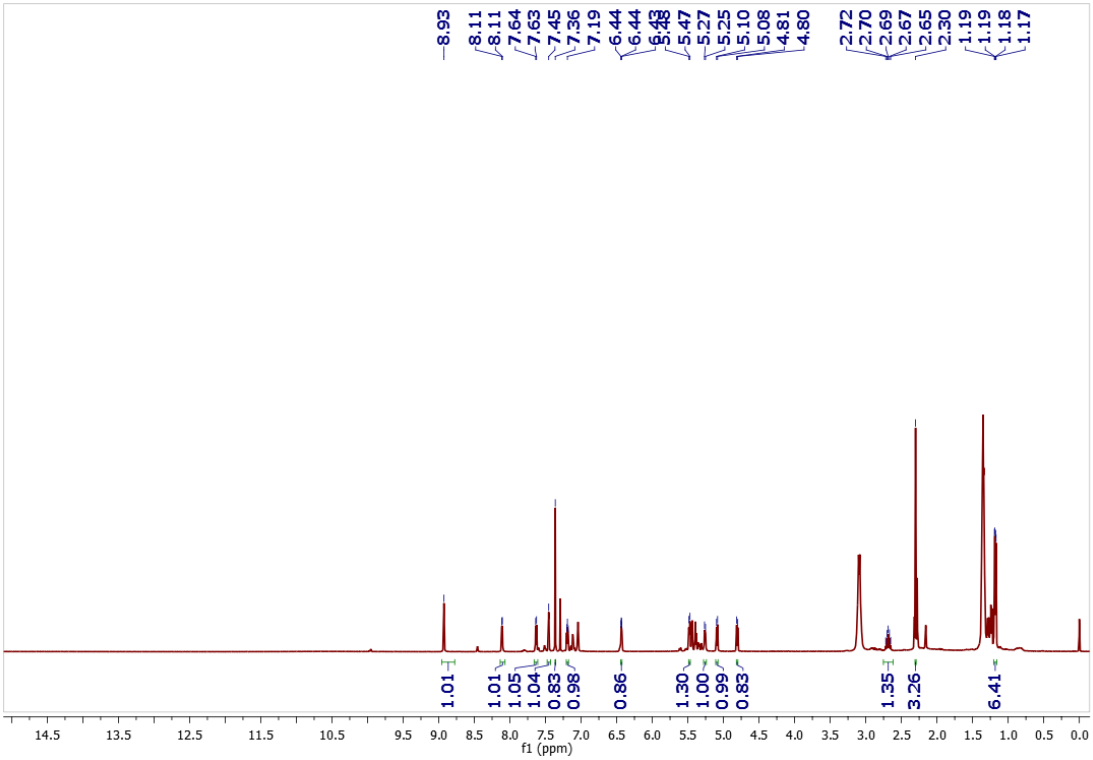

Figure S6. ^{1}H NMR spectrum of Complex **3**

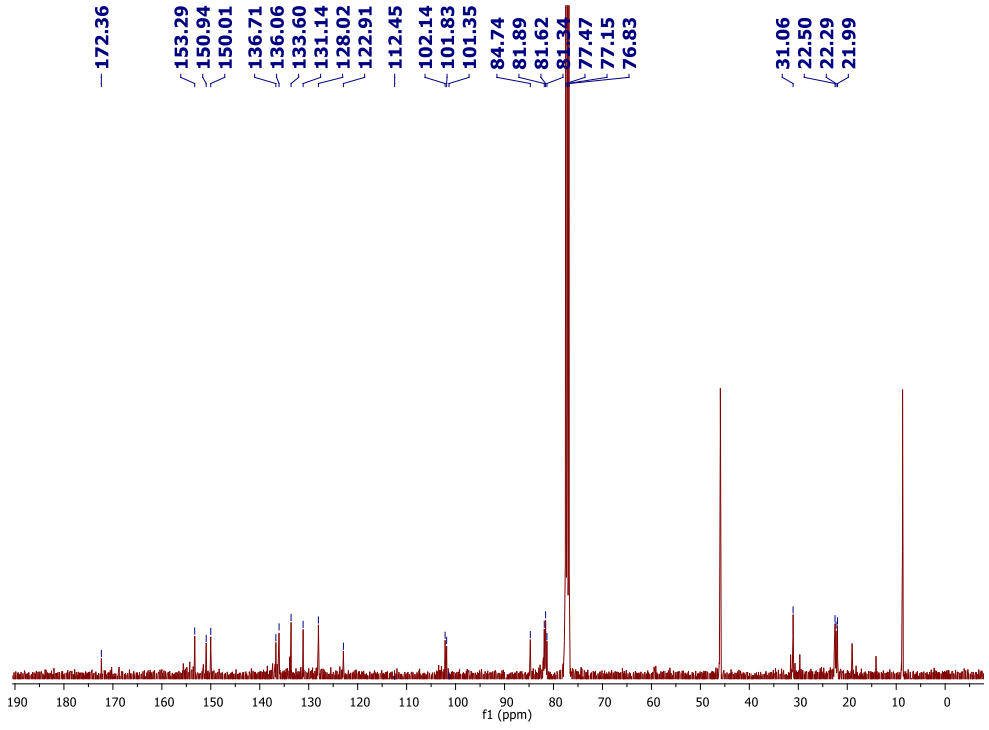

Figure S7. ^{13}C NMR spectrum of Complex **1**

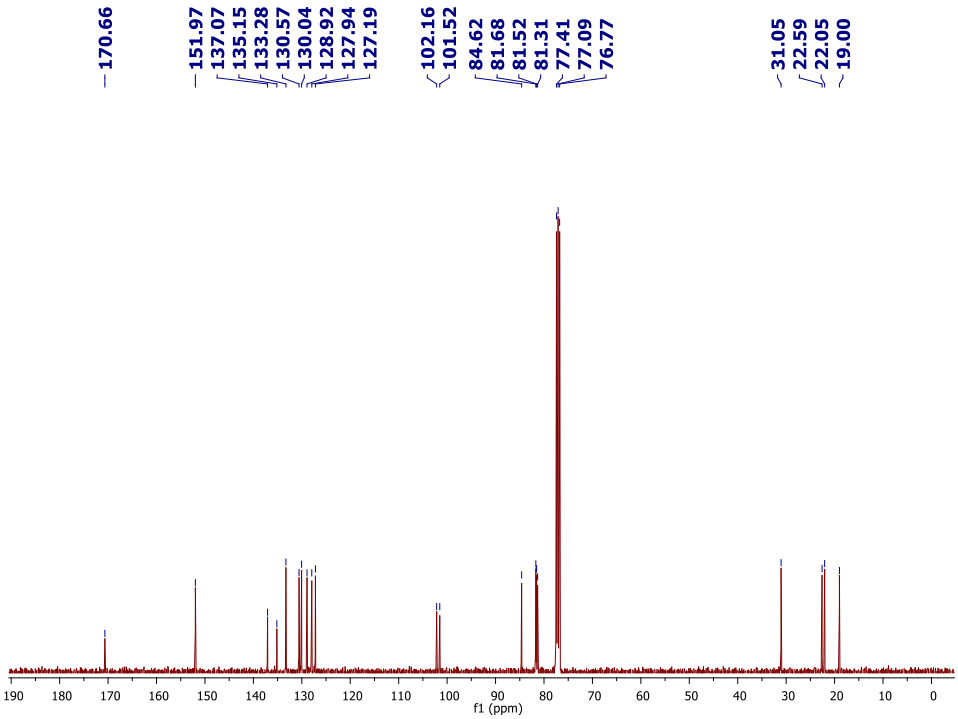

Figure S8. ^{13}C NMR spectrum of Complex **2**

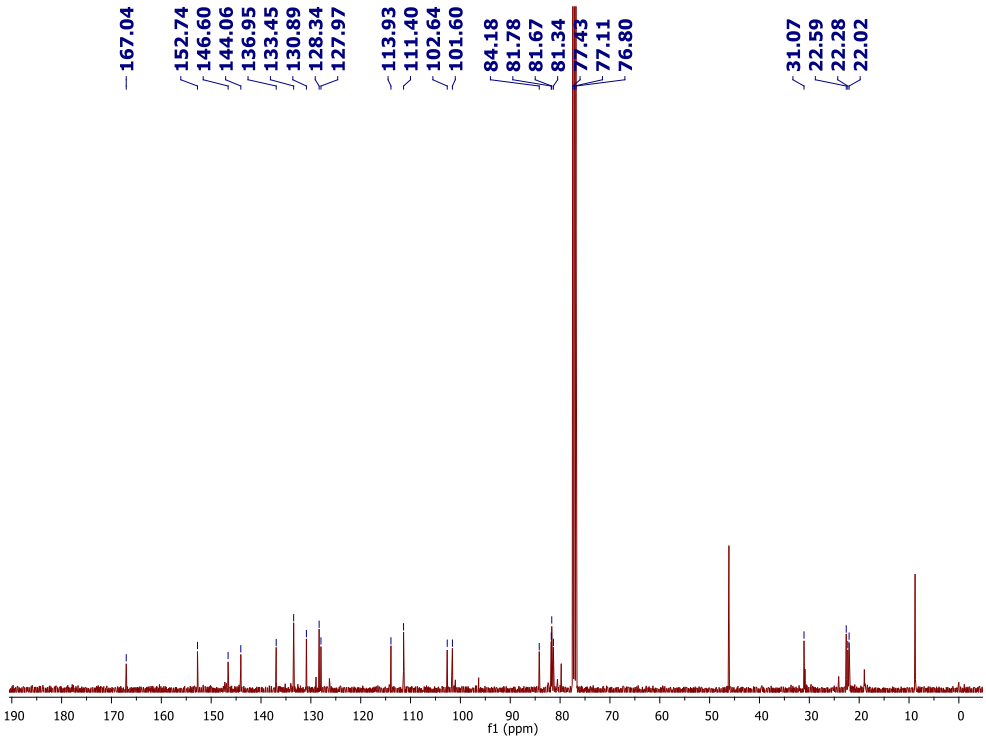

Figure S9. ^{13}C NMR spectrum of Complex **3**

HR-MS spectra of the complexes(1-3)

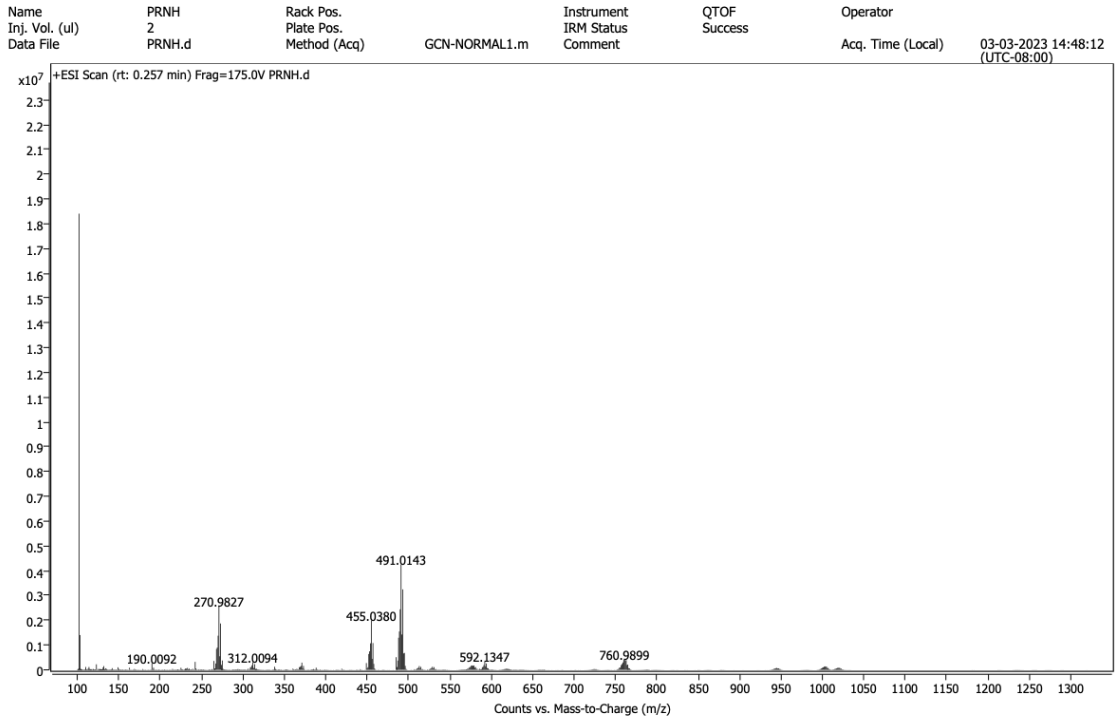

Figure S10. ESI-MS spectrum of [Ru(L1)(η^6 -p-cymene)Cl] (1) in acetonitrile; m/z: 491.0143;$[M+H]^+$

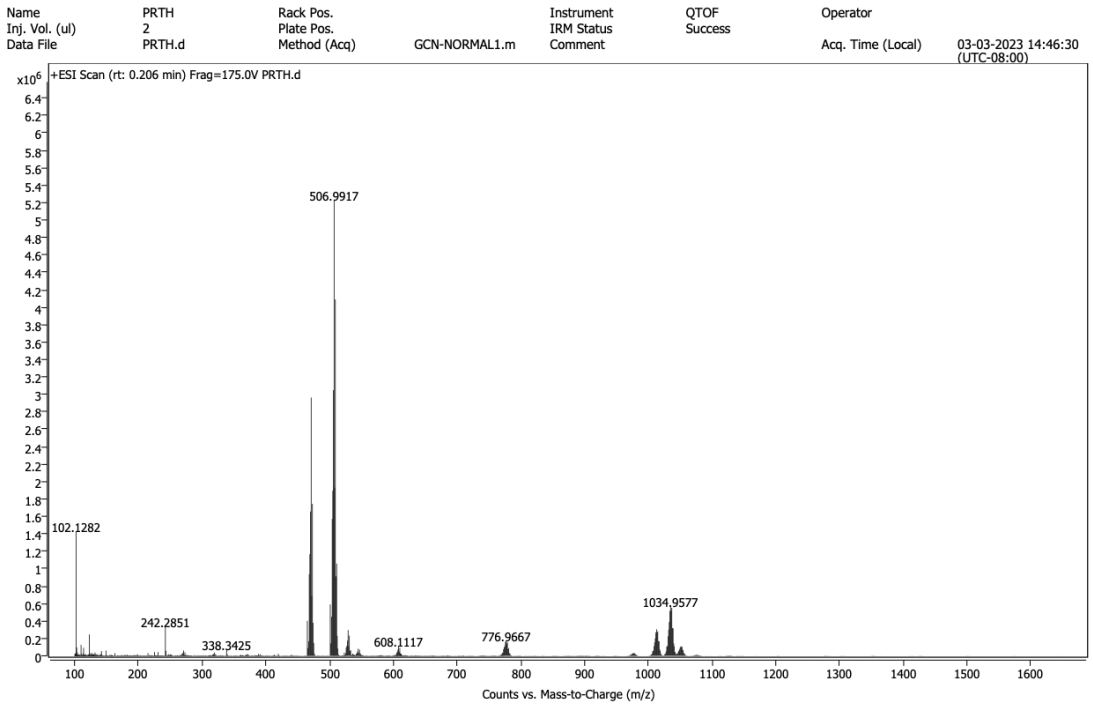

Figure S11. ESI-MS spectrum of [Ru(L2)(η^6 -*p*-cymene)Cl] (2) in acetonitrile; m/z: 506.9917;
$[M+H]^+$

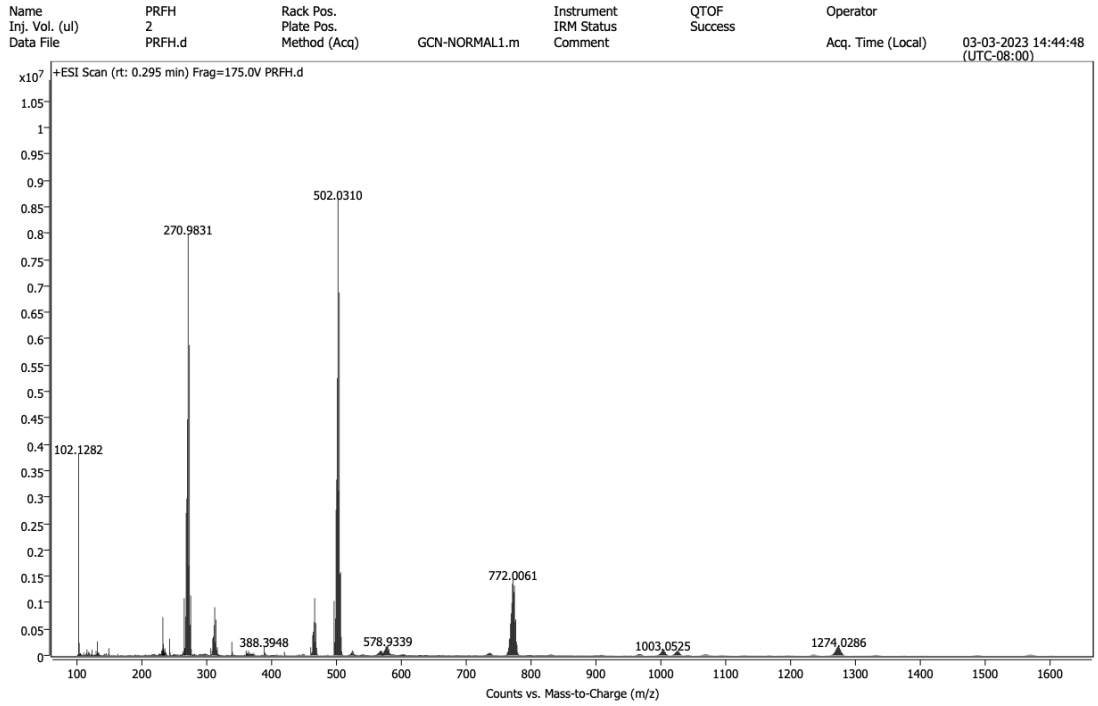

Figure S12. ESI-MS spectrum of [Ru(L3)(η^6 -*p*-cymene)Cl] (3) in acetonitrile; m/z: 502.0310;$[M+H]^+$

Table S1: Crystal data and structure refinement for complex **2**

	Complex **2**
Empirical formula	$C_{20}H_{21}ClN_2ORuS_2$
Formula weight	506.052
Temperature/K	273.15
Crystal system	monoclinic
Space group	$P2_1/n$
a/Å	7.7107(12)
b/Å	19.424(3)
c/Å	14.081(2)
α/°	90
β/°	96.242(5)
γ/°	90
Volume/Å^3	2096.5(6)
Z	4
ρ_{calc}g/cm^3	1.603
μ/mm^{-1}	1.087
F(000)	1021.0
Crystal size/mm^3	0.29 × 0.18 × 0.15
Radiation	Mo Kα (λ = 0.71073)
2Θ range for data collection/°	5.1 to 60.24
Index ranges	$-10 \le h \le 10$, $-26 \le k \le 27$, $-19 \le l \le 19$
Reflections collected	23607
Independent reflections	5991 [R_{int} = 0.0298, R_{sigma} = 0.0245]
Data/restraints/parameters	5991/0/247
Goodness-of-fit on F^2	1.099
Final R indexes [I>=2σ (I)]	R_1 = 0.0349, wR_2 = 0.0923
Final R indexes [all data]	R_1 = 0.0404, wR_2 = 0.0989
Largest diff. peak/hole / e Å^{-3}	0.72/-0.72

Table S2: Selected bond lengths (Å) and angles (°) for the complex 2

Bond lengths		**Bond angles**	
N(1)-Ru(1)	2.101(2)	O(1)-Ru(1)- N(1)	76.55(9)
O(1)-Ru(1)	2.0589(18)	N(1)-Ru(1)-C(2)	131.33(11)
Cl(1)- Ru(1)	2.4070(7)	O(1)-Ru(1)-C(2)	151.54(10)
N(1)-N(2)	1.393(4)	C(16)-O(1)-Ru(1)	112.11(18)
C(16)- N(2)	1.310(4)	O(1)-Ru(1)-Cl(1)	84.60(5)
C(16)-O(1)	1.287(3)	N(1)-Ru(1)-Cl(1)	84.89(6)
C(15)-N(1)	1.296(4)	O(1)-C(16)-N(2)	125.9(3)
C(6)-Ru(1)	2.183(2)	C(15)-N(1)-N(2)	113.2(2)
*Centroid$_{\text{Ru-}p\text{-cymene}}$	1.678	C(16)-N(2)-N(1)	111.0(2)

Stability studies

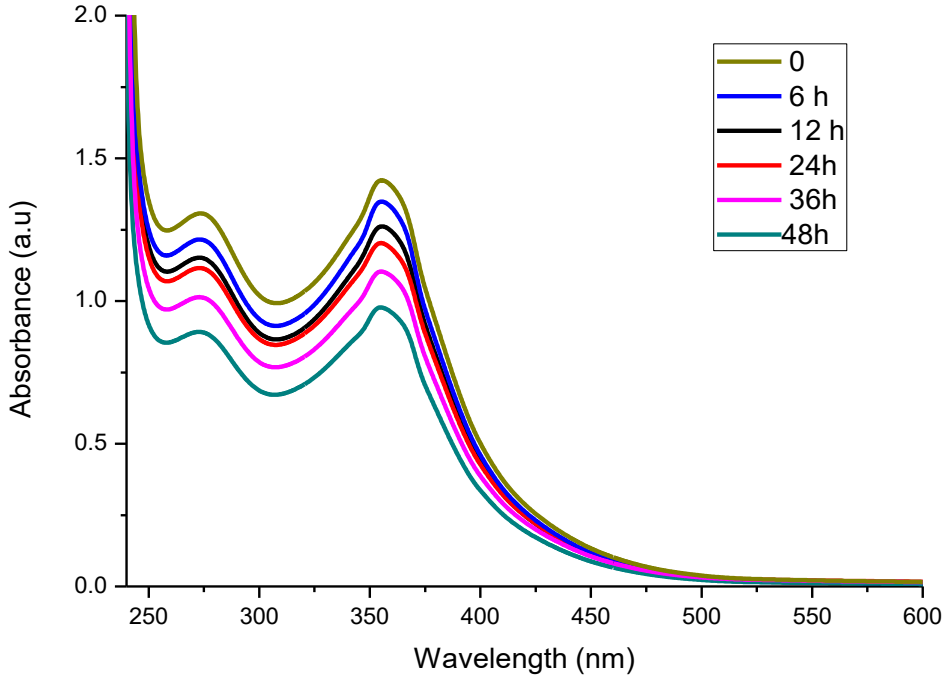

Figure S13. Stability study for Complex **1**

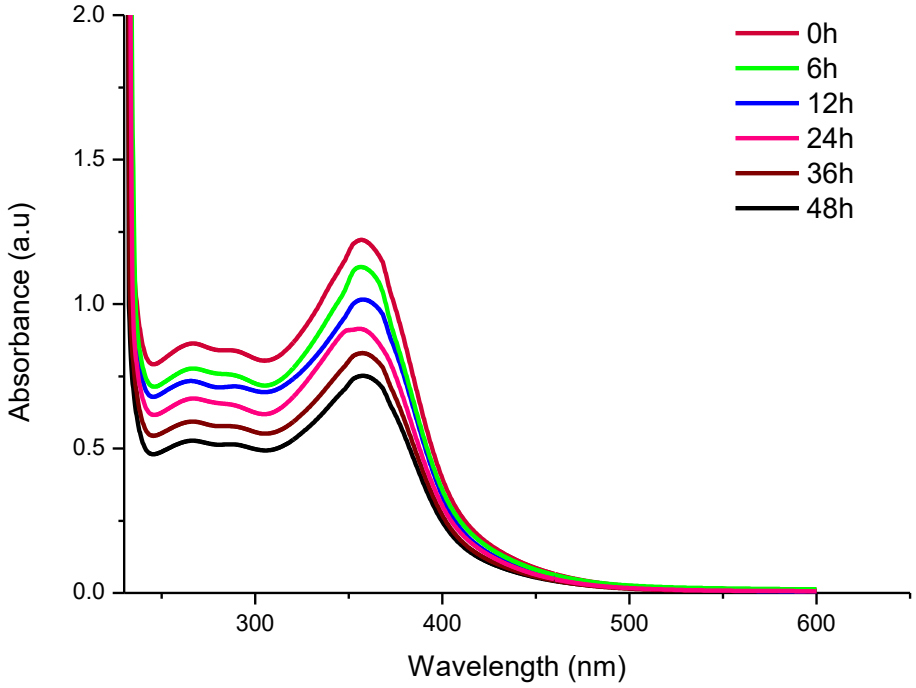

Figure S14. Stability study for Complex **2**

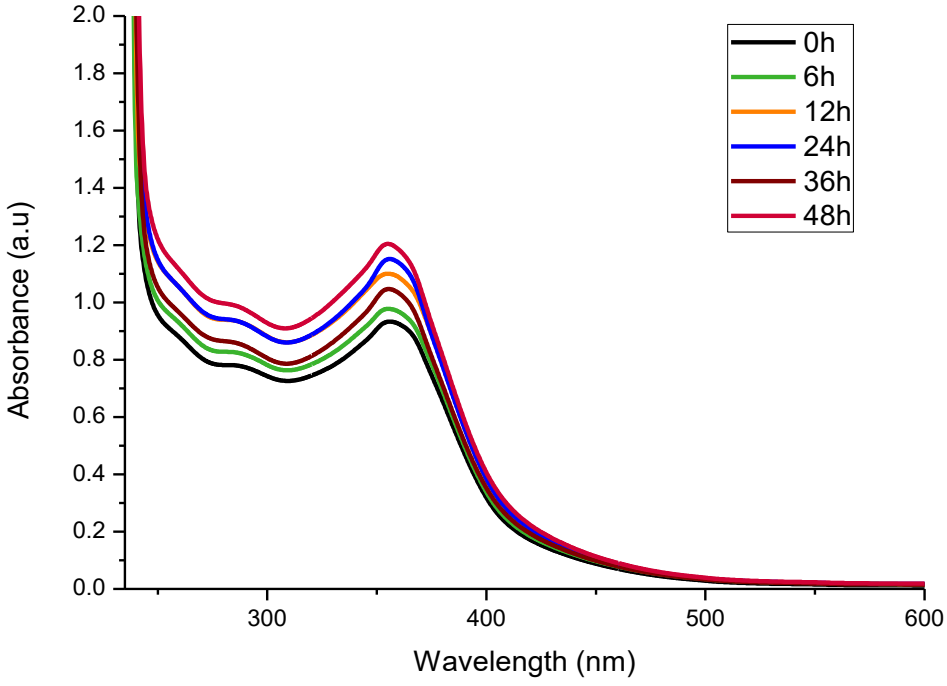

Figure S15. Stability study for Complex **3**

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