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Synthesis, Characterization and Biological evaluation of Ruthenium Flavanol Complexes against breast cancer

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Abstract

Four Ru(II) DMSO complexes (**M1R**-**M4R**) having substituted flavones *viz.* 3-Hydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one (**HL1**), 3-Hydroxy-2-(4-nitrophenyl)-4H-chromen-4-one (**HL2**), 3-Hydroxy-2-(4-dimethylaminophenyl)-4H-chromen-4-one (**HL3**) and 3-Hydroxy-2-(4-chlorophenyl)-4H-chromen-4-one (**HL4**) were synthesized and characterized by elemental analysis, IR, UV-Vis, ^{1}H NMR spectroscopies and ESI-MS. The molecular structures of the complexes were investigated by integrated spectroscopic and computational techniques (DFT). Both ligands as well as their complexes were screened for anticancer activities against breast cancer cell lines MCF-7. Cytotoxicity was assayed by MTT [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay. All ligands and their complexes exhibited significant cytotoxic potential of 5 – 40 µM concentration at incubation period of 24 h. The cell cytotoxicity increased significantly in a concentration-dependent manner. In this series of compounds, **HL2** (IC_{50} 17.2 µM) and its complex **M2R** (IC_{50} 16 µM) induced the highest cytotoxicity.

Keywords: Ru(II); DMSO; Flavones; Cytotoxicity; DFT

1. Introduction

During the last three decades, platinum-based drugs have played a crucial role in cancer chemotherapy [1]. The *cis*-[$PtCl_2(NH_3)_2$] complex, known as *cis*-platin is the most widely used drug in anticancer therapy [2,3]. In recent years, the antitumor properties of ruthenium(II) compounds are gaining considerable importance and a (chloroimidazole) ruthenium(III) complex had entered in phase I clinical study [4a]. The first ruthenium complex investigated for anticancer activity was *fac*-[$RuCl_3(NH_3)_3$] [4b]. This compound was not pursued much further because its poor solubility precludes it from adequate formulation as a drug [5]. The anticancer activity of ruthenium(II) complex, *cis*-[$RuCl_2(DMSO)_4$] (DMSO = dimethyl sulfoxide), is well-known and had been compared with *cis*-platin [6a]. Ruthenium anticancer *viz.* ruthenium DMSO and ofloxacin ruthenium bipyridyl complexes based drugs [6b-e] have no side effects when compared with platinum based drugs which is having relatively more side effects such as nephrotoxicity and neurotoxicity as a result its use has been limited [7]. The metallo-drugs based on ruthenium are often identified as less toxic and capable of overcoming the resistance induced by platinum drugs in cancer cells [8]. Due to these properties several ruthenium based anticancer agents, ruthenium-DMSO complexes and the coordination complexes of ruthenium-chloro-DMSO containing heterocyclic ligands *viz*. NAMI, have proved their potential in the treatment of cancer cells [9]. The *cis*-[$Ru(DMSO)_4Cl_2$] has been widely used as a precursor for the synthesis of Ru(II) complexes by displacement of DMSO molecules with variety of ligands [10]. Ruthenium drugs are gaining importance in clinic due to their low toxicity and specific activity than the current metallo-drugs [11]. Furthermore, ruthenium complexes appear to penetrate into the tumor cells where they bind effectively with DNA [12]. The two ruthenium compounds, ImH [$Ru(im)CI_5$] [13], NAMI-A (im = imidazole) and Ru (III) compound indazolium *trans*-[tetrachlorobis(1H-indazole) ruthenate (III)] KP1019 [14] were the first ruthenium-based anticancer drugs which entered into clinical trials [15]. Also, many other compounds that include ruthenium center have been developed and tested [16] *viz.*, trans-(IndH)[$Ru(ind)_2Cl$ (Ind=indazole), mer-$Ru(tpy)Cl_3$(terpy=2,2’,6’,2”-terpyridine [17, 18], ImH[$Ru(im)CI_5$]andImH[$Ru(im)_2CI_4$] were found to be active as cytostatic drugs [19, 20]. Flavones are the yellowish polyphenolic compounds belonging to class flavonoids which exist in plants and are having secondary metabolites, 2-phenyl benzopyran functionality. They are

potential synthetic targets as they display wide range of biological activities [21-23], including antibacterial [24], antioxidant [25], anti-inflammatory [26-28], antiallergic [29-31] and antiestrogenic [32] activities. Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrone rings, and are classified into, flavones, flavonols, flavanones, isoflavones and anthocyanidins. Flavonols are the most abundant flavonoids in foods [33]. The flavones also known as anthoxanthins are yellow pigments, which is responsible for colour of flavones show antioxidant properties [34]. It may be possible that ruthenium and other transition metals can form coordination complexes with flavones having different substituents at *para*- position. Because of the differences in the electronic properties of these *para*-substituents the anticancer activities of the resulting complexes against breast cancer may be tuned.

With these viewpoints and in the quest of new ruthenium(II)DMSO flavonol complexes having potential against breast cancer cell lines herein we report the synthesis, characterization and biological evaluation of four new ruthenium(II)DMSO complexes with substituted flavonols against breast cancer.

2. Experimental

2.1 Materials and measurements

All chemicals were purchased from Aldrich-sigma, Himedia and E. Merck and used without further purification. Solvents were purified and dried according to standard procedure [35]. IR spectra were recorded on Perkin–Elmer AC-1 spectrometer and ^{1}H NMR spectra were recorded in DMSO-d_6 on Bruker Avance IIIHD spectrometer at 400 MHz using TMS as an internal reference. Elemental analysis were performed on Exeter analytical Inc.‘‘Model CE-440 CHNS analyzer’’. ESI-MS spectrometry were performed on JEOL SX 102/DA-6000. Electronic absorption spectra were recorded on LT 2900–UV-Vis spectrophotometer. Conductance measurements were carried by using Esico model-1601. All biological activities were carried out from Department of Zoology, University of Lucknow.

2.2 Cell culture

MCF-7 is an estrogen receptor positive breast cancer cell line derived from pleural effusion and is the most commonly used cell line for screening of anticancer breast agents. MCF-7 cells obtained from cell repository-NCCS Pune (Job No. 390) were cultured in Eagle's minimal essential medium (MEM, Himedia) with NEAA, 1.5 g/L $NaHCO_3$, 1 mM sodium pyruvate and 0.01 mg/mL insulin with 10% fetal calf serum (Himedia).

2.3 Cell cytotoxicity (MTT assay)

The antiproliferative activities of the compounds were determined using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Himedia] assay according to Sharma *et al.* [36]. In brief, 1×10^4 cells/well were seeded in 100 µL complete MEM (minimal essential medium) in each well of 96-well culture plates and incubated for 24 h at 37°C in a CO_2 incubator. Compounds were diluted to the desired concentrations in culture medium and added to the wells with respective vehicle control. After 24 h of compounds supplementation, 10 µL of MTT reagent (5 mg/mL) was added in each well and the plates were further incubated for 4 h at 37°C until purple formazan crystal produced. Supernatant was then removed from each well and crystals were solubilized in 100 µL of DMSO reagent. The plate was left for 10 min at 37°C and absorbance was then recorded at 540 nm by a microplate reader (BIORAD Model 680). For each condition, at least three independent experiments were performed [36, 37]. The percentage antiproliferative activities were calculated by using the formula-

$$\%\ Cell\ cytotoxicity = \frac{(OD\ of\ control - OD\ of\ treated)}{(OD\ of\ control)} \times 100$$

The plot of % cell cytotoxicity versus compound concentration was applied to calculate the IC_{50}.

2.4 Analysis of cellular DNA content and detection of apoptotic cells

MCF-7 cells were plated into 6-well plate at density 1×10^6 cells/mL and treated with a concentration of IC_{50} value of highly active compounds **HL1** and **M1R** for 24 h in 5% CO_2 incubator and 37°C temperature. After 24 h incubation, the cultured cells were harvested and washed with cold PBS and fixed in 70% ethanol, treated with RNAse A (10 mg/mL) and stained with propidium iodide, PI (10 µg/mL) for 30 min at room temperature in dark. The PI fluorescence of individual nuclei was measured using a FACS-Calibur cytometer (BD Biosciences, Heidelberg, Germany). Data were analyzed with the Cell Quess Pro V5.2.1 software (BD Biosciences).

2.5 Statistical analysis

The results are presented in mean ± SE. The unpaired t-test was used to compare the differences between parameters at different concentrations. The one way analysis of variance (ANOVA) was used to compare the differences within parameters among concentrations. The Bonferroni pair wise comparison test was applied to compare the differences between concentrations for a parameter if found significant in ANOVA. The p-value <0.05 was considered to be as significant.

2.6 Synthesis: 3-Hydroxy-4-substituted flavonols HL1-HL4 and their metal complexes M1R-M4R

Step A: Chalcones were synthesized by Claisen-Schmidt reaction by previously reported methods. The synthesis of compounds were carried out in dilute ethanolic sodium hydroxide solution by cyclization and Claisen- Schmidt condensation of 2-hydroxyacetophenone with different 4-substituted benzaldehyde [38,39,40] at room temperature. The equimolar ratio of 2-hydroxy acetophenone (10 mmol, 1 mL) with 4-substituted benzaldehyde (10 mmol, 1 mL (for **HL1**)/ 1.52 g (for **HL2**)/ 1.50 g (for **HL3**)/ 1.41g (for **HL4**)) in 30 mL of ethanol were stirred and 40% NaOH solution (10 mL) was added dropwise and the mixture was stirred for 4 days. After stirring for 4 days the solution was diluted with water, acidified with 10% HCl, and kept overnight. The crude products were filtered, washed with diethyl ether and recrystallized in ethanol to get the desired chalcones. Chalcones (1 mmol; 0.254 g for **HL1**)/ 0.269 g (for **HL2**)/ 0.267 g (for **HL3**)/ 0.258 g (for **HL4**)) were dissolved in ethanol (15 mL) and stirred and 10% (15 mL) NaOH was added. To the resulting mixtures 30% H_2O_2 (10 mL) was added and the precipitate was recovered by addition of dichloromethane. The obtained flavonols were recrystallized using ethanol.

3-Hydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one (HL1): Colour: Reddish Brown; Yield (55%, 0.140 g); m. p. 135^{o}C; IR (KBr/cm^{-1}): 1684 $\nu_{C=O}$, 3500 ν_{OH}, 1283 δ_{OH}, 1591 $\nu_{C=C}$; ^{1}H NMR (400 MHz, DMSO-d_6, 25^{o}C, TMS): δ 10.67 (s, 1H, OH), δ 6.99-7.73(multiplet, Ar-H), δ 2.4 (s, 6H, OCH_3); For $C_{16}H_{12}O_4$; ESI/MS (268). Calcd: C 71.64, H 4.48, Found: C 72.03, H 4.72.

3-Hydroxy-2-(4-nitrophenyl)-4H-chromen-4-one (HL2): Colour: Dark Brown; Yield (60%, 0.169 g); m. p. 138^{o}C; IR (KBr/cm^{-1}): 1693 $\nu_{C=O}$, 3366 ν_{OH}, 1235 δ_{OH}, 1520 $\nu_{C=C}$; ^{1}H NMR (400 MHz, DMSO-d_6, 25^{o}C, TMS): δ 10.86 (s, 1H, OH), δ 7.16-7.50 (Ar-H); For $C_{15}H_9NO_5$; ESI/MS (283). Calcd: C 63.60, H 3.18, N 4.95 Found: C 63.10, H 3.32, N 4.68.

3-Hydroxy-2-(4-dimethylaminophenyl)-4H-chromen-4-one (HL3): Colour: Brown; Yield (62%, 0.187 g); m. p. 140^{o}C; IR (KBr/cm^{-1}): 1692 $\nu_{C=O}$, 3475 ν_{OH}, 1273 δ_{OH}, 1568 $\nu_{C=C}$; ^{1}H NMR (400 MHz, DMSO-d_6, 25^{o}C, TMS): δ 13.35 (s, 1H, OH), δ 7.14-7.50 (Ar-H), δ 3.68 (s, 6H, CH_3).; For $C_{17}H_{15}NO_3$; ESI/MS (281). C 72.60, H 5.34, N 4.98 Found: C 72.96, H 5.52, N 4.74.

3-Hydroxy-2-(4-chlorophenyl)-4H-chromen-4-one (HL4): Colour: Yellow; Yield (65%, 0.154 g) ; m.p. 135^{o}C; IR (KBr/cm^{-1}): 1699 $\nu_{C=O}$, 3423 ν_{OH}, 1209 δ_{OH}, 1566 $\nu_{C=C}$; ^{1}H NMR (400 MHz,

DMSO-d_6, 25^{o}C, TMS): δ 10.57 (s, 1H, OH), δ 7.22-7.73 (Ar-H); For $C_{15}H_9O_3Cl$; ESI/MS (272). C 66.18, H 3.31, Found: C 65.83, H 3.62.

Step B: $[Ru(DMSO)_4Cl_2]$ was prepared according to the method reported by Evans [10]. The synthesis of $[Ru(DMSO)_4Cl_2]$ was carried out by mixing of $RuCl_3.3H_2O$ and 1 mL of dimethylsulphoxide and heating the mixture for 1 h. The yellow crystals of $[Ru(DMSO)_4Cl_2]$ was washed with ether then was used as such. All the complexes were prepared under nitrogen atmosphere. The mononuclear complexes were synthesized by drop-wise addition of ethanol solution of ligands **HL1-HL4** (1 mmol; 0.268 g (**HL1**)/ 0.283 g (**HL2**)/ 0.281 g (**HL3**)/ 0.272 g (**HL4**)) to the ethanolic solution of $[Ru(DMSO)_4Cl_2]$ (1 mmol; 0.484 g). After complete addition of ligand one drop of triethylamine was added to the resultant mixture and whole mixture was refluxed with stirring for 7 h. The completion of reaction was monitored through TLC. After 7 h the contents were cooled to room temperature and filtered. To the filtrate a saturated methanolic solution of sodium nitrate was added. The brown precipitate thus obtained was filtered and washed with water, methanol followed by ethanol/ diethyl ether and then purified by column chromatography over alumina using CH_3CN as eluent. The molecular formula of the complexes are $[Ru(DMSO)_2(L1)_2]$ $2NaNO_3.2H_2O$ (**M1R**); $[Ru(DMSO)_2(L2)_2].2NaNO_3.H_2O$ (**M2R**); $[Ru(DMSO)_2(HL3)_2](NO_3)_2.2H_2O$ (**M3R**)**;** and $[Ru(DMSO)_2(L4)_2].2NaNO_3.5H_2O$ (**M4R**). The conductivity measurements of metal complexes show that **M1R**, **M2R** and **M4R** are non-electrolytic but **M3R** is electrolytic in nature.

$[Ru(DMSO)_2(L1)_2]$ $2NaNO_3.2H_2O$ (M1R): Colour: Reddish brown; Yield (65%, 0.375g); m. p. 360^{o}C; IR (KBr/cm^{-1}): 1604 $\nu_{C=O}$, 3422 ν_{OH}, 1254 δ_{OH}, 1528 $\nu_{C=C}$, 1111 $\nu_{S=O}$, 512 ν_{Ru-O}, 1384 ν_{NO3}; ^{1}H NMR (400 MHz, DMSO-d_6, 25^{o}C, TMS): δ 6.90-8.70 (Ar-H), δ 3.60 (s, 6H, CH_3, DMSO-S), δ 2.50 (CH_3, 6H, DMSO-O), δ 1.27 (s, OCH_3); For $RuNa_2C_{36}H_{36}N_2S_2O_{18}$, ESI/MS (979). Calcd.: C 44.13, H 3.68, N 2.86, S 6.54, Found C 44.38, H 3.82, N 2.86, S 6.38.

$[Ru(DMSO)_2(L2)_2]$ $2NaNO_3.H_2O$ (M2R): Colour: Dark brown; Yield (55%, 0.278 g); m. p. 360^{o}C; IR (KBr/cm^{-1}): 1635 $\nu_{C=O}$, 3435 ν_{OH}, 1174 δ_{OH}, 1508 $\nu_{C=C}$, 1174 $\nu_{S=O}$, 509 ν_{Ru-S}, 1384 ν_{NO3}; ^{1}H NMR (400 MHz, DMSO-d_6, 25^{o}C, TMS): δ 10.00 (s, OH), δ 7.00-8.32 (Ar-H), δ 3.00 (s, 6H, CH_3, DMSO-S), δ 2.50 (s, 6H, DMSO, CH_3) ; For $RuNa_2C_{34}H_{32}N_4S_2O_{19}$.; ESI/MS (1009). Calcd: C 40.44, H 3.17, N 5.55, S 6.34, Found: C 40.37, H 3.23, N 5.38, S 6.57.

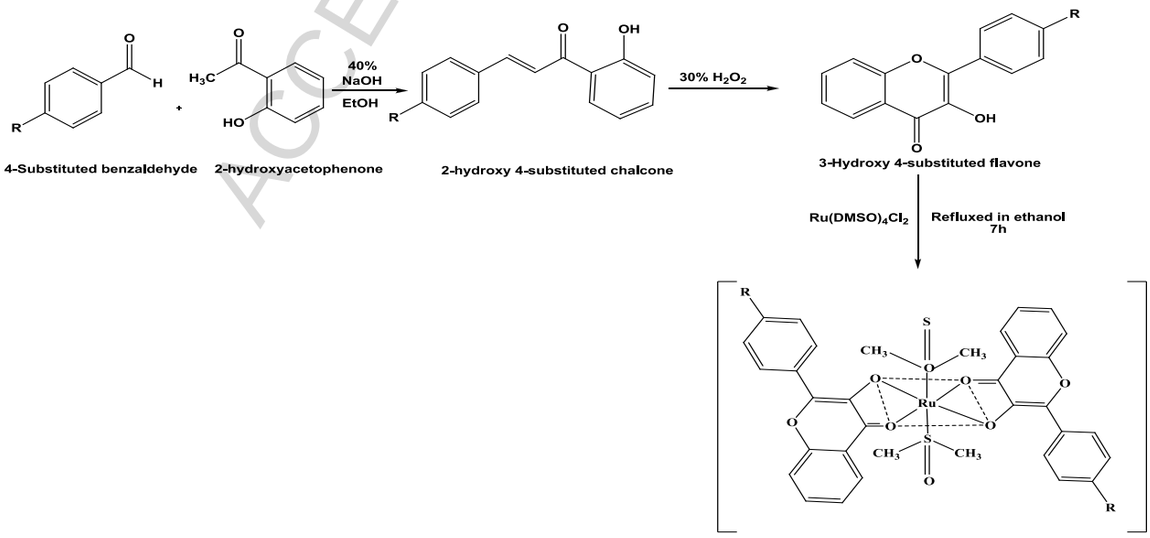
$[Ru(DMSO)_2(HL3)_2](NO_3)_2.2H_2O$ (M3R): Colour: Brownish black; Yield (70%, 0.365g); m. p. 360^{o}C; IR (KBr/cm^{-1}): 1605 $\nu_{C=O}$, 3431 ν_{OH}, 1259 δ_{OH}, 1508 $\nu_{C=C}$, 1111 $\nu_{S=O}$, 509 ν_{Ru-S}, 1384

ν_{NO3}; ^{1}H NMR (400 MHz, DMSO-d_6, 25 ^{o}C, TMS): δ 11.92 (s, OH), δ 13.14 (OH), δ 6.64-8.52 (Ar-H), δ 3.02 (d, DMSO-S), δ 2.82 (DMSO-O), δ 2.50 ($N(CH_3)_2$, 6H, d), δ 1.21 (s, OCH_3); For $RuC_{38}H_{46}N_4S_2O_{16}$.; ESI/MS (979). Calcd.: C 46.58, H 4.70, N 5.72, S 3.27, Found C 46.28, H 4.46, N 5.54, S 3.42.

$[Ru(DMSO)_2(L4)_2]2NaNO_3.5H_2O$ (M4R): Colour: Brown; Yield (65%, 0.378 g); m. p. 360^{o}C; IR (KBr/cm^{-1}): 1686 $\nu_{C=O}$, 3418 ν_{OH}, 1174 δ_{OH}, 1543 $\nu_{C=C}$, 1091 $\nu_{S=O}$, 548 ν_{Ru-S}, 1384 ν_{NO3}; ^{1}H NMR (400 MHz, DMSO-d_6, 25 ^{o}C, TMS): δ 7.17 -7.94 (Ar-H), δ 3.30 (s, 6H, DMSO-S), δ 2.5 (s, 6H, DMSO-O); For Ru $NaC_{34}H_{40}N_2S_2O_{19}Cl$.; ESI/MS (1060) Calcd.: C 38.53, H 3.78, N 2.64, S 3.02, Found C 38.25, H 3.74, N 2.34, S 3.47.

2.7 Computational details

In order to ascertain the nature of molecules and their electronic transition, density functional theory (DFT) and time-dependent DFT calculations were performed. Optimized molecular geometries were calculated using the B3LYP exchange-correlation functional [41, 42]. The LANL2DZ basis set for Ru and 6-31G** basis set for other atoms were used for geometry optimization. The optimized structures of the compounds were used for molecular orbital analyses as well as for the time-dependent density functional theory (TD-DFT) calculations using polarised continuum model (PCM) [40]. All the calculations were performed using Gaussian 09 program [43].



R=-OCH_3 (**M1R**);-NO_2(**M2R**); -NMe_2(**M3R**), -Cl(**M4R**)

Scheme 1. Synthetic routes for the ligands and metal complexes.

3. Results and discussion

3.1 Optimized geometry of complexes

The optimized geometry of the metal complexes of Ru (II) are shown in Figure 1. The immediate geometry around the Ru(II) in all the complexes are satisfied by two flavone ligands through bidentate mode as well as by two DMSO ligands in monodentate mode there by leading to a distorted octahedral geometry (Fig. 1). The calculated bond lengths and bond angles as presented in Table 1 are comparable with the reported metrical parameters of the similarly structured complexes [44]. In all the four complexes the Ru-O bond lengths are in good agreement with the previously reported values [45].

Table 1. Optimized geometrical parameters for the metal complexes (bond length (Å) and bond angle (°).

Parameters	**M1R**	**M2R**	**M3R**	**M4R**
Ru-S	2.251(2.282)	2.282 (2.282)	2.280(2.282)	2.255(2.282)
Ru-O1	2.115 (2.134)	2.110 (2.134)	2.115 (2.134)	2.114 (2.134)
Ru-O2	2.118 (2.087)	2.117 (2.087)	2.197 (2.087)	2.118 (2.087)
Ru-O3	2.196	2.188	2.127	2.193
Ru-O4	2.120 (2.087)	2.116 (2.087)	2.119 (2.087)	2.120 (2.087)
Ru-O5	2.132 (2.134)	2.127 (2.134)	2.133 (2.134)	2.130(2.134)
Ru-S-O6	120.32	118.30	120.00	119.56
Ru-O1-C	112.65	113.17	112.98	112.88
Ru-O2-C	111.98	112.47	111.85	112.20
Ru-O3-S	113.98	115.37	113.55	114.57
Ru-O4-C	112.53	143.95	112.33	112.76
Ru-O5-C	111.82	112.26	111.65	112.05

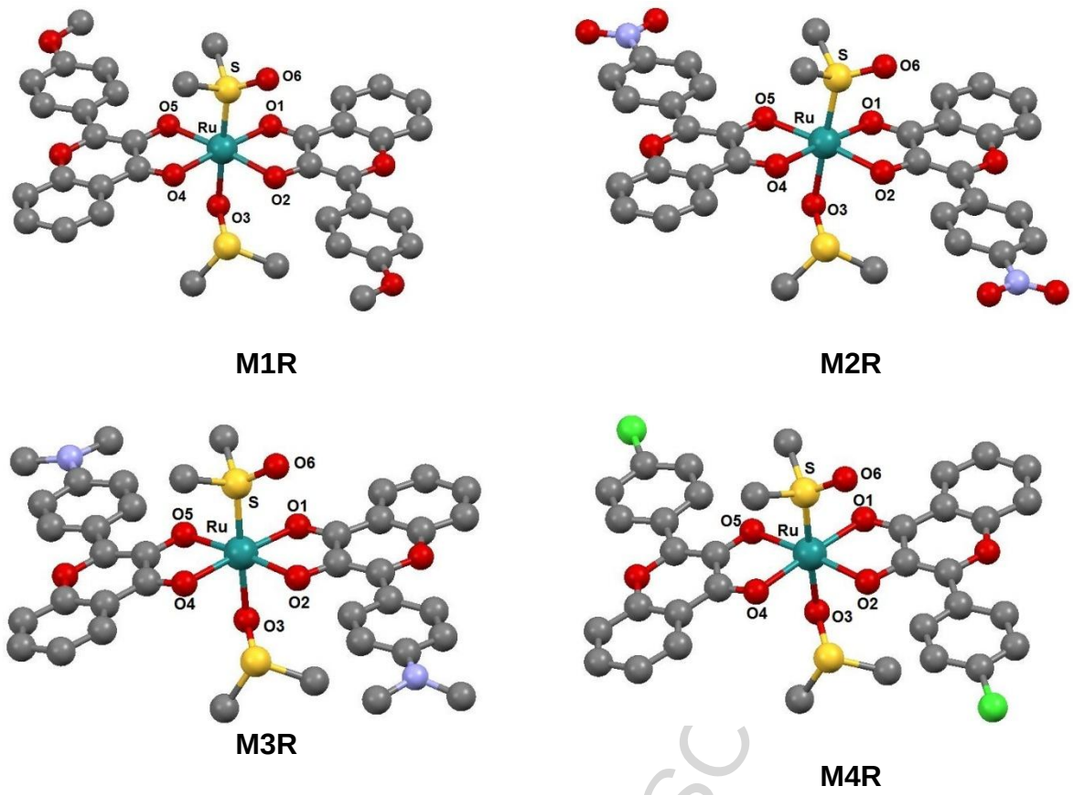


Fig. 1. Perspective view of the optimized geometry of metal complexes (Colour legends: Red Oxygen; Yellow Sulfur; grey carbon; dark green ruthenium and light green chlorine).

Table 2. IR (in cm^{-1}) spectroscopic data for the ligands and Ru(II) complexes.

Compounds	$\nu_{C=O}$	ν_{O-H}	δ_{O-H}	$\nu_{C=C}$	$\nu_{S=O}$	ν_{Ru-S}
HL1	1684	3500	1283	1591	-	-
HL2	1693	3366	1235	1520	-	-
HL3	1692	3475	1273	1568	-	-
HL4	1699	3423	1209	1566	-	-
M1R	1604	3422	1254	1528	1111	512
M2R	1635	3435	1174	1508	1174	509
M3R	1605	3431	1259	1508	1111	509
M4R	1686	3418	1174	1543	1091	548

3.2 Infrared spectroscopy

The IR spectra of the ligands displayed ν(C=O) bands at 1684, 1693, 1692 and 1699 cm^{-1} for **HL1, HL2, HL3** and **HL4,** respectively, which on coordination to the Ru(II) center gets slightly

shifted to lower energy to 1604, 1635, 1605 and 1686 cm^{-1} for **M1R, M2R**, **M3R** and **M4R**, respectively there by confirming the coordination of C=O part of the ligand to ruthenium center. In the IR spectra of **M3R** the major shift to lower energy (50 cm^{-1}) observed for hydroxyl group δ_{OH} indicated its coordination with the Ru(II) center. However, observed vibrations for the hydroxyl group in other metal complexes (**M1R, M2R** and **M4R**) were due to lattice water [46] which was further supported by mass spectrometry and ^{1}H NMR spectroscopy. Furthermore, in **M1R** the presence of peaks at 557 and 512 cm^{-1} corresponding to ν (Ru-O), supported that hydroxyl group of flavone is coordinated. The coordinated DMSO molecule show band in the range 1174-1091 cm^{-1} which was assigned to ν_{S-O} stretching of S-coordinated DMSO molecule which is significantly shifted from the corresponding ν_{S-O} of free DMSO observed at 1050 cm^{-1} [46b]. Additionally presence of single band also indicates the presence of coordinated DMSO molecule in *trans*-position. The appearance of peak at 1024, 1015, 1016 and 1015 cm^{-1} for **M1R, M2R, M3R** and **M4R**, respectively is due to O-bonded DMSO molecule and lowering in stretching frequency might be due to the lowering in bond order of S=O group. This is further supported by Ru-S mode, which appears as two bands at 1111 and 1024 cm^{-1} [47]. The magnitude of separation confirms a symmetric bidentate coordination for all Ru complexes. In the solid state the nitrate stretch and deformation bands were also found in all complexes at ~1384 and 830 cm^{-1}. The comparative IR spectroscopic data for the ligands and complexes are presented in table 2.

3.3 NMR spectroscopy

The ^{1}H NMR spectra display aromatic protons in the range 6.0 to 8.0 ppm and which indicates the coordination of two ligands. The two sharp peaks in the range 3.39-2.50 ppm, confirmed coordination of DMSO ligand through DMSO-S and DMSO-O, respectively [48]. In **M1R**, **M2R** and **M4R**, the absence of signal at ~11.00 ppm indicates the deprotonations of OH proton in corresponding flavone ligands. However, in **M3R** the appearance of two singlets at 11.92 and 13.14 ppm corresponding to the OH proton of flavones indicate that two flavones ligands are coordinated to ruthenium center. The comparative ^{1}H NMR spectroscopic data for the ligands and complexes are presented in table 3.

Table 3. ^{1}H NMR (in ppm) spectroscopic data for the ligands and Ru(II) complexes.

Compounds	OH	Ar-H	DMSO-S	DMSO-O
HL1	10.67	6.99-7.73	-	-
HL2	10.86	7.16-7.50	-	-
HL3	13.35	7.14-7.50	-	-
HL4	10.57	7.22-7.73	-	-
M1R	-	6.90-8.70	3.60	2.50
M2R	13.14	7.00-8.32	3.00	2.50
M3R	-	6.64-8.52	3.02	2.82
M4R	-	7.17 -7.94	3.30	2.5

3.4 Mass spectrometry (ESI-MS)

In ESI-MS for **M1R**, of $[Ru(DMSO)_2(L1)_2].2NaNO_3.2H_2O$ showed molecular ion peak at (m/z 980), ascribed at 979, further the fragmentation pattern showed loss of lattice water molecules from coordination sphere, which can be ascribed to 943 for $[Ru(DMSO)_2(L1)_2].2NaNO_3$ (m/z at 944), and loss of $2NaNO_3$ also ascribed to 775 (calc.) for $[Ru(DMSO)_2(L1)_2]$ at (m/z 776). All other complexes showed similar loss of water and sodium nitrate from outside of the coordination sphere (Fig. S4). In the mass spectrum of **M2R** the peak corresponding to $[Ru(DMSO)_2(L2)_2].2NaNO_3.H_2O$ was observed at m/z 1010 (calcd. 1009); $[Ru(DMSO)_2(HL2)_2].2NaNO_3$ showed peak at m/z 990 (calcd. 991); the signal for $[Ru(DMSO)_2(L2)_2]$ observed at m/z 823 (calcd. 823); $[Ru(L2)_2]$ at m/z 667 (calcd. 667). The mass spectrum for **M3R** $[Ru(DMSO)_2(HL3)_2](NO_3)_2.2H_2O$ showed peak at m/z 978 (calcd. 979); $[Ru(DMSO)_2(HL3)_2](NO_3)_2$ fragment was observed at m/z 820 (calcd. 818); $[Ru(DMSO)(HL3)_2]$ fragment was observed at m/z 663 (calcd. 662). In **M4R** $[Ru(DMSO)_2(HL4)_2].2NaNO_3.5H_2O$ shows a peak at m/z 1059 (calcd. 1060); $[Ru(DMSO)_2(L4)_2].2NaNO_3$ fragment was observed at m/z 968 (calcd. 970); $[Ru(DMSO)_2(L4)_2]$ fragment was observed at m/z 801 (calcd. 802).

3.5 Electronic Absorption Spectroscopy

The electronic absorption spectra recorded in acetonitrile for all the complexes consisted of well-defined bands in the range at 200-600 nm (Table 4**)**. However, a number of significant

differences between the ligand and metal complexes are arising due to the functionalization at 4′-position of 3-hydroxy flavones (Fig. S3). The absorbance at 250-300 nm, can be assigned to π-π* transition of the ligands. In addition, a series of strong ligand centered absorption were observed which can be attributed to the DMSO ligands. Also the absorption observed in the range 428-550 nm is due to metal to ligand charge transfer. To gain further insight into the nature of electronic absorption spectra TD-DFT calculations were carried out. The electron density distributions for the selected frontier molecular energy levels for the complexes are shown in Fig. S5. In order to locate the electronic transitions, HOMO must be localized on the donor site and LUMO should be on the acceptor site. The electron densities of the highest occupied molecular orbitals are mainly located with contribution of 50 and 52% respectively and the ligand character is about 35%. In the case of **M2R**, HOMO, HOMO-1 and HOMO-2 are entirely localized on the Ruthenium centre and contribution of about 80% and 20% of the flavonol ligands. In the LUMOs of **M1R-M4R** the contribution from the flavonols ligand was observed to be maximum.

Table 4. Electronic absorption spectroscopic data for the ligands and Ru(II) complexes.

Compound	**Wavelength λ_{max} (nm) ε_0**
HL1	203 (16400), 220 (2500), 237 (26400), 255 (30700), 270 (55300), 314 (53200), 355 (82500), 432 (24300).
HL2	222 (4000), 236 (4100), 256 (5200), 273 (19700), 290 (2500), 480(9600)
HL3	245 (2000), 277 (30800), 340 (34300), 455 (27800).
HL4	270 (7300), 310 (1800), 340 (1500), 368 (16900), 308 (27700), 347 (32000), 434 (65000).
M1R	220 (13300), 252 (3280), 269 sh (3360), 311 (16700), 330 (2900), 420 (2550), 430 (1600), 440 sh (1470).
M2R	310 (3200), 430 (2700), 510 sh (1200)
M3R	270 sh (2700), 400 sh (6900),
M4R	272 (32300), 310 (19500), 350(1800), 430 (4400), 463 (2200) shoulder.

3.6 Cell cytotoxicity

The complex **M1R** was found to be most antiproliferative with IC_{50} value of 16 µM followed by the activity of **HL1**, **M2R**, **HL2**, **M3R** and **HL3** with the IC_{50} value of 17.2, 28.0, 29.5, 32.1 and 35.4 µM, respectively. However, **M4R** and **HL4** were found to be least effective with the IC_{50} value of 36.2 and 38.4 µM, respectively (Table 5).

Table 5. Concentration for 50% inhibition**,** required to inhibit cancer cell proliferation by 50 % of Ru(II) DMSO complexes along with p-substituted 3-hydroxy flavones.

Compounds	**HL1**	**M1R**	**HL2**	**M2R**	**HL3**	**M3R**	**HL4**	**M4R**
Cell inhibition IC_{50} (µM)	17.2	16.0	29.5	28.0	35.4	32.1	38.4	36.2

Similarly, dose dependent percentage antiproliferative activities for all compounds were presented in the form of Mean±SE. The data were presented in three different ways of comparisons such as between compounds and within concentration, within compounds and between concentrations and among all the compounds (Table 6) and metal complexes compared with standard drug *cis*-platin and other ruthenium complexes. **HL1** and **M1R** were found to be more effective ($p<0.0001$) as compared to all other compounds at each concentration while **M1R** was most effective among all ligands and complexes. After the coordination of ligands with Ru(II) metal, all the metal complexes were screened to be more active than their corresponding ligands. The compounds **HL1** and **M1R** exhibited maximum antiproliferative activities as compared to the other all compounds in a dose dependent manner. The ligands showed activities in the order **HL1>HL2>HL3>HL4** and their Ru(II) complexes which were observed to be more active than ligands follows the activity order **M1R>M2R>M3R>M4R**. Table 6 and Figure 2 are representing the all possible comparisons in between compounds and their concentrations. The compounds which revealed less than 50% antiproliferative activities can be considered suitable DOSE in an *in vitro* analysis as comparable to IC_{50}. The 5 and 10 µM dose of all the compounds showed less than 50% antiproliferative activities. While at 20 µM **HL2, HL3, HL4** and their complexes exhibited less than 50% activities as well at 30 µM concentrations only **HL3** and

HL4 show less than 50% activities. All other doses of compound revealed cytotoxicity when compared to IC_{50}.

Table 6. Antiproliferative activities of all compounds and their comparative analysis

Compounds	**Concentrations in µM(Mean±SE)**				
	5	**10**	**20**	**30**	**40**
HL1	16.41±0.39[1,2]	28.55±0.37[1,2]	59.10±0.75[1,2]	63.44±0.57[1,2]	66.62±0.55[1,2]
M1R	19.25±0.65[1]	31.37±0.48[1]	62.92±0.93	64.66±0.52	67.91±0.64[1,2]
p-value$	0.01*	0.004*	0.02*	0.16	0.18
HL2	15.51±0.89[1]	27.12±0.47[1]	42.03±2.56[1,2]	50.32±0.56[1,2]	55.85±0.97[1,2]
M2R	18.33±1.25[1]	30.87±0.82[1]	43.55±0.84[1]	52.16±0.79[1,2]	61.06±0.63[1,2]
p-value$	0.12	0.007*	0.59	0.11	0.004*
HL3	12.64±0.48[1]	22.76±0.94[1]	34.30±2.29[1,2]	44.94±0.68[1,2]	54.67±0.75[1,2]
M3R	16.11±0.82[1]	26.83±0.71[1]	39.22±1.40[1,2]	47.72±0.48[1,2]	58.91±0.59[1,2]
p-value$	0.01*	0.01*	0.12	0.02*	0.004*
HL4	8.54±0.43[1,2]	16.75±0.97[1,2]	27.20±0.44[1,2]	38.92±1.25[1,2]	52.53±0.69[1,2]
M4R	11.00±0.81[1]	18.83±0.29[1]	29.39±0.88[1,2]	41.72±1.20[1,2]	57.44±1.12[1]
p-value$	0.04*	0.09	0.07	0.16	0.01*

*Unpaired t-test between compounds and within concentration, *Significant ($p<0.05$), [1]Significant within compounds and between concentrations ($p<0.0001$), [2]Significant ($p<0.0001$) between HL1, HL2, HL3, HL4 and M1R, M2R, M3R, M4R*

The antiproliferative activity of **HL1** was significantly higher ($p<0.05$) at concentration of 5 µM (16.41±0.39), 10 µM (28.55±0.37) and 20 µM (59.10±0.75) than **M1R** as well as rest of the ligands and complexes at same concentration. The activity of **HL1** was significantly effective ($p<0.0001$) in comparison to all other compounds. However, the activities corresponding to **M1R** recorded at 5, 10, 20 µM concentrations were significantly higher as compared to **HL1** and other compounds ($p<0.0001$).

The effect of **HL2** was significantly higher ($p<0.05$) at concentration 10 µM (27.12±0.47) and 40 µM (55.85±0.97) than **M2R**. The effect of **HL2** and **M2R** was recorded significantly different between all the concentrations ($p<0.0001$) (Table 6 and Figure 2). Similar observation was found for **HL4** and **M4R** (Table 5 and Figure 2). There was no significant difference between **HL2**, **HL3** and **HL4** at concentration 5. However, HL1 (16.41±0.39) was significantly ($p<0.001$) higher than HL4 (8.54±0.43) at concentration 5. Similar observation was found at concentration 10. No difference observed among **M1R, M2R, M3R** and **M4R** at concentration 5. The **HL1** (59.10±0.75) values was significantly ($p<0.001$) higher than **HL2** (42.03±2.56), **HL3** (34.30±2.29) and **HL4** (27.20±0.44) at concentration 20. Almost similar observation was found at the concentration 30 and 40 for all the parameters and **M1R, M2R, M3R** and **M4R**. The antiproliferative activities of the synthesized complexes show relatively lower IC_{50} values than NAMI-A and other related ruthenium-DMSO complexes [49-51]. The results indicate that ruthenium-DMSO flavone complexes may prove to be prospective candidates as anticancer agents against breast cancer.

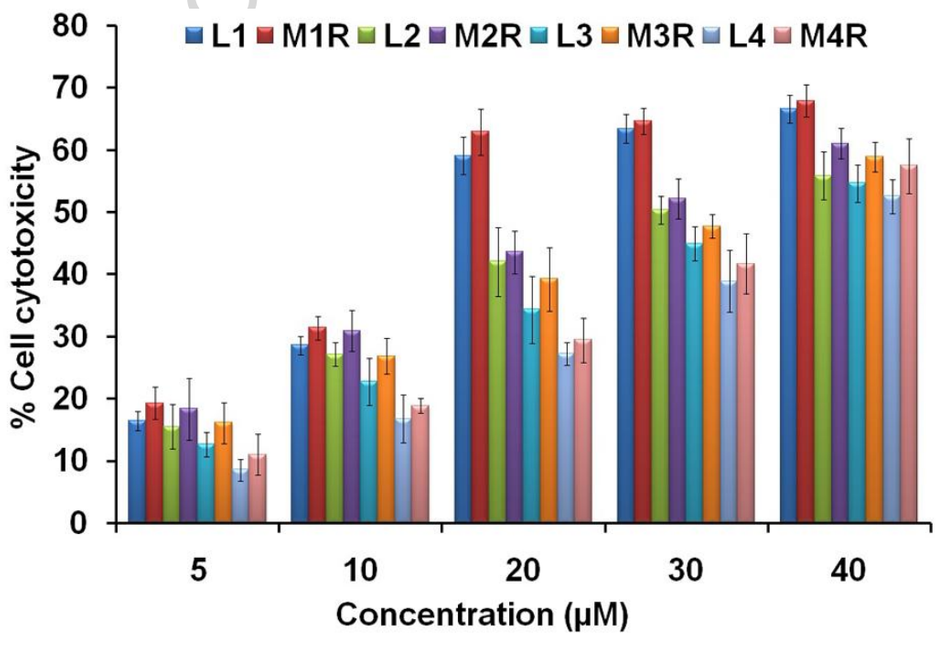
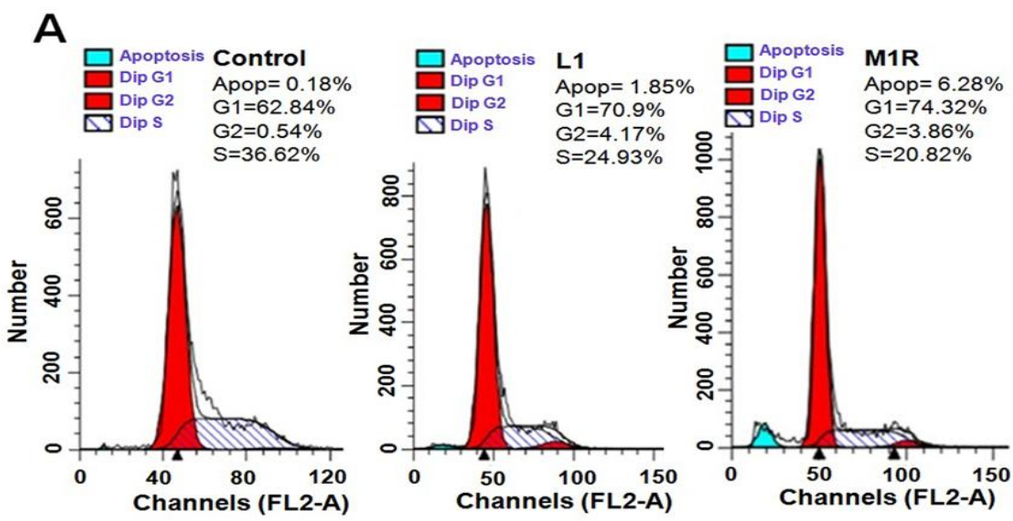


Fig. 2. In vitro activity of ligands and their respective Ru (II) complexes against Human Breast Cancer Cell Line MCF-7 measured by a 24 hr. MTT assay as described in the experimental section.Values are expressed as means ± SE of at least three independent value.

3.7 Cell cycle assay

Analysis of cell-cycle phase distribution was carried out to study the antiproliferative mechanism of most active compound **HL1** and **M1R** on a single dose of 16µM. **Figure3B** shows the G1 checkpoint was interfered with both **HL1** and **M1R**. However, G1 phase was dramatically increased and S phase was reduced, when treated with **M1R**. These results indicated that **M1R** induced G1 arrest by inhibiting DNA synthesis in S phase and cell apoptosis. G1 and G2 phases of cell cycle are the major check points, have an important role in cell cycle progression [52-53]. Also, all activities were compared with previously reported antiproliferative activity of ofloxacin and Ru (II) complexes on HeLa cell line [54-58] which indicated that Ru(II) complex containing more aromatic ring will be exhibiting more effective antiproliferative activity [54]. In the same vein, our results also indicated that the complexes reported herein show more effective antiproliferative activity due to the presence of *para*-substituted aromatic rings on flavonols and can be more persuasive drug than the previously reported drugs [59-60]. The antiproliferative activity of **M1R** was investigated in MCF-7 breast cancer cells and was found to be more active than *cis*-platin against GI50 of T-47D breast cancer [61-62].



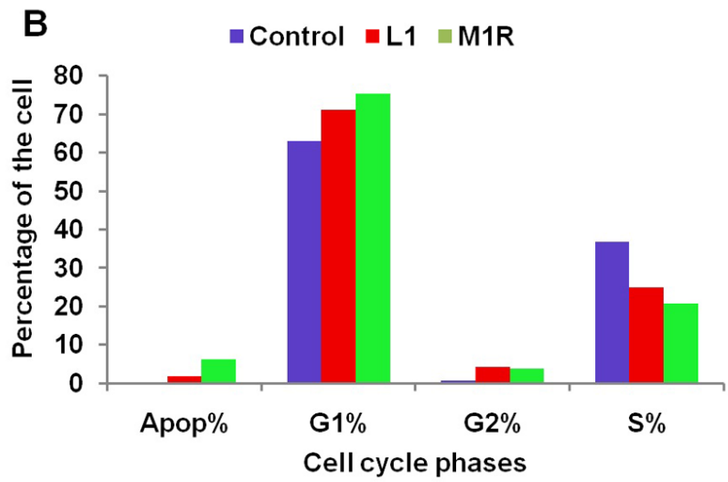


Fig. 3. Cell cycle distribution of MCF-7 cells treated with IC_{50} value of compounds L1 and M1R for 24 h **(A)** Original cell cycle diagram **(B)** Quantitative distribution of percentage of cells in different phases of cell cycle.

4. Conclusion

Investigation indicated that among the ruthenium(II) complexes of *para*-substituted-3-hydroxy flavones reported herein, the complex **M1R** comprising of $–OCH_3$ group was found to be most antiproliferative with IC_{50} value of 16 µM followed by the activity of **HL1, M2R, HL2, M3R** and **HL3**. **M4R** and **HL4** were found to be least effective. Analysis of cell-cycle phase distribution was carried out to study the anti-proliferative mechanism of most active compound **HL1** and **M1R** on a single dose of 16 µM. They show the G1 check point was interfered with both **HL1** and **M1R**. This inhibits G1 arrest by inhibiting DNA synthesis in S Phase and cell apoptosis. It can be concluded that the ruthenium(II) complexes having *para*- substituted 3-hydroxy flavones with DMSO as ancillary ligands can become potential candidate as anticancer agents in future. The antiproliferative activities of the synthesized complexes show relatively lower IC_{50} values than NAMI-A and other related ruthenium-DMSO complexes. The results indicate that ruthenium-DMSO flavone complexes may prove to be prospective candidates as anticancer agents against breast cancer.

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Synthesis, Characterizations and Biological evaluation against breast cancer of Ruthenium Flavanol Complexes

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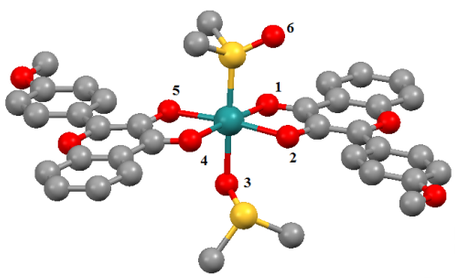
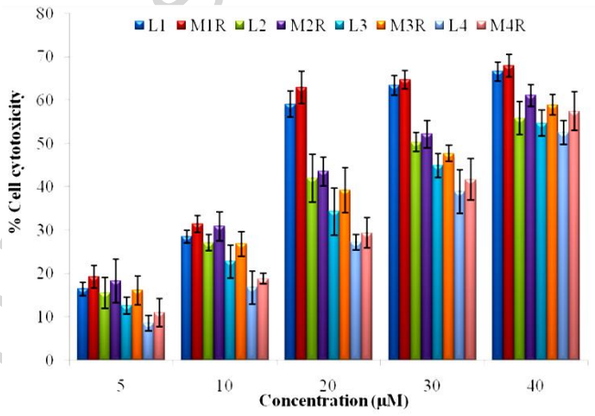
Graphical abstract

The Ru**(**II**)**-DMSO complexes containing different 4-substituted 3-hydroxy flavones synthesized which showed significant activity against breast cancer cell line, MCF-7.



M1R



Research highlights:

- Ru(II)-DMSO flavonol complexes synthesized.
- The complexes were characterized using integrated physico-chemical technique
- The complexes were tested against breast cancer cell lines.