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A novel Ru(II) complex derived from hydroxydiamine as a potential antitumor agent: Synthesis and Structural Characterization

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ABSTRACT

A novel Ru(II)-Schiff base complex has been synthesized by the interaction of ligand, 1,3-bis{[(*E*)-(2-chlorophenyl) methylidene]amino}-2-propanol, L with $[RuCl_2(PPh_3)_3]$. The structure of ligand, L has been determined on the basis of X-ray diffraction while the geometry of the Ru(II) complex has been ascertained by FT-IR, ^{1}H, $^{13}C\{^{1}H\}$, $^{31}P\{^{1}H\}$ NMR and UV–vis studies. The *in vitro* antitumor activity of these compounds was assessed by examining their ability to inhibit cell proliferation against human breast and pancreatic cancer cell lines. The results show a dose-dependent anti-proliferative effect and induction of apoptotic cell death in both the cancer cell lines, thus indicating pharmacological significance of Ru(II) complex against cancer.

Introduction. Metal complexes and their application in medicine have been extensively investigated since the discovery of the antineoplastic activities of cisplatin by Rosenberg in 1960 [1,2]. Cisplatin or [*cis*-diamminedichloroplatinum(II)], a bifunctional reagent, is highly effective for the treatment of various types of tumors viz., testicular, ovarian, bladder, small cell lung, head and neck cancers etc. [3]. Currently, cisplatin, carboplatin and oxaliplatin are some of the most effective chemotherapeutic agents in clinical use [4,5]. In spite of great efficacy of cisplatin, carboplatin and oxaliplatin against ovarian, bladder and testicular cancer, these drugs display limited activity against some of the most common tumors, such as colon and breast cancer. In addition, a variety of adverse effects viz., nausea, bone marrow suppression and kidney toxicity and acquired resistance are observed in patient receiving cisplatin chemotherapy [6]. Therefore, there is a need for new approaches that are purposefully designed to circumvent these drawbacks. Efforts are focused to develop novel platinum and non-platinum based antitumor drugs to improve clinical effectiveness to reduce general toxicity and broaden the spectra of activity. In the field of non-platinum compounds exhibiting anticancer properties, ruthenium complexes are found very promising alternative to platinum, showing activity on tumors which developed resistance to cisplatin or in which cisplatin inactive [7] thus indicating that it may be a strong candidate to form a basis for rational anticancer drug design [8]. In addition, some chemical properties, such as rate of ligand exchange, range of accessible oxidation states and ability of ruthenium to mimic iron in binding to certain biological molecules make these compounds well suited for medicinal applications as an alternative to platinum antitumor drugs in the treatment of cancer cells resistant to cisplatin and its analogues justifying further development of the novel and interesting metal complexes [9]. Interestingly, two Ru(III) complexes, namely, *trans*-$[RuCl_4(Im)(DMSO)]$(ImH) (NAMI-A) and *trans*-$[RuCl_4(Ind)_2]$(IndH) (KP1019) have successfully completed phase I clinical trials [10–12]. The majority of ruthenium compounds evaluated for anticancer activity are in +3 oxidation state. It has been reported that Ru(III) is less active and is reduced *in vivo* to more active ruthenium(II) complexes, a process favoured in the hypoxic environment of a tumour [13]. However, it should be noted that Ru(II) complexes generally exhibit a low toxicity [14]. The first ruthenium complex evaluated of this kind was [Ru(benzene)(metronidazole)Cl_2] which presented a higher activity compared to the antitumour drug metronidazole itself [15]. In recent years, Ru-arene complexes, most of which are coordinatively unsaturated compounds in +2 oxidation states, are considered to be promising candidates for anticancer drug design and have also shown excellent *in vitro* results revealing high selectivity and low general toxicity [16,17]. Herein, we report the synthesis and characterization of novel *cis*-Ru(II) complex derived from Schiff base ligand, 1,3-bis{[(*E*)-(2-chlorophenyl) methylidene]amino}-2-propanol. Crystal structure of ligand, 1,3-bis {[(*E*)-(2-chlorophenyl)methylidene]amino}-2-propanol has been determined by single crystal XRD. Cytotoxic studies were carried out

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Table 1
Crystal data and structure refinement for ligand, L.

Empirical formula	C17 H16 Cl2 N2 O	
Formula weight	335.22	
Temperature	293(2) K	
Wavelength	0.71073 Å	
Crystal system	Orthorhombic	
Space group	P c a 21	
Unit cell dimensions	a = 7.2500(7) Å	α = 90°.
	b = 7.6569(9) Å	β = 90°.
	c = 29.039(3) Å	γ = 90°.
Volume	1612.0(3) $Å^3$	
Z	4	
Density (calculated)	1.381 Mg/m^3	
Absorption coefficient	0.405 mm^{-1}	
F(000)	696	
Crystal size	0.42 × 0.32 × 0.10 mm^3	
Theta range for data collection	2.81 to 26.35°.	
Index ranges	−9<=h<=8, -9<=k<=9, -36<=l<=33	
Reflections collected	5954	
Independent reflections	2765 [R(int) = 0.0348]	
Completeness to theta = 26.35°	99.9%	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.9606 and 0.8482	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	2765 / 1 / 203	
Goodness-of-fit on F^2	1.180	
Final R indices [I > 2sigma(I)]	R1 = 0.0520, wR2 = 0.1436	
R indices (all data)	R1 = 0.0694, wR2 = 0.1511	
Absolute structure parameter	−0.12(13)	
Largest diff. peak and hole	0.246 and −0.229 $e.Å^{-3}$	

to examine its potential against human breast and pancreatic cancer cell lines in order to evaluate its pharmacological significance.

Experimental section. All chemicals were used as received. The preparations were carried out in Schlenk tubes under inert atmosphere using standard Schlenk line techniques. ^1H, $^{13}C\{^1H\}$ and $^{31}P\{^1H\}$ NMR spectra were recorded in deutrated chloroform using JEOL 400 spectrometer. FT-IR spectra were obtained as KBr pellet on Perkin Elmer 621 spectrophotometer while the electronic spectrum was recorded in dichloromethane on Pharmacia LKB-Biochem, UV–vis spectrophotometer at room temperature. Elemental analyses were determined on Perkin Elmer Analysator 2400.

Breast cancer cells (MDA-MB-231) and pancreatic cancer cells (BxPC3) were obtained from ATCC (Manassas, V A). The cell lines were grown and maintained as monolayer cell culture in DMEM (Invitrogen, Carlsbad, CA) with 10% fetel bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2–humidified atmosphere at 37 °C.

A methanolic solution of 2-chlorobenzaldehyde (2 mml) was added dropwise to the methanolic solution of 1,3-diaminopropanol (1 mml). The reaction mixture was refluxed for 2 h resulting in to a yellow colored solution. The resulting solution was concentrated to 1 ml followed by addition of 15 ml of n-hexane to cause precipitation. The precipitate was collected and recrystallised in CH_2Cl_2-n-hexane. After couple of days, yellow crystals suitable for single crystal XRD were obtained.

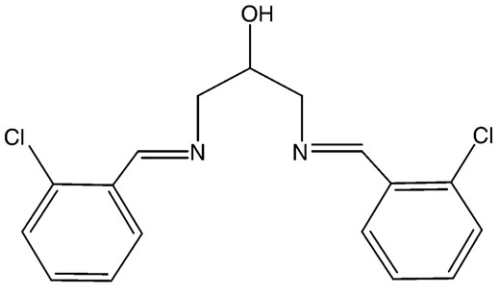
Yield 85%, Color, yellow, mp 108 °C, ^1H NMR ($CDCl_3$): ∂ (ppm) 3.14-4.28 (m 4H -CH2), 4.2 (s 1H-CH), 4.46 (br 1H, -CHOH), 7.25-7.36 (m, Ar-H), $^{13}C\{^1H\}$NMR: ∂ (ppm) 65.00 (−CH2), 70.97 (−CHOH), 159.89 (−CH=N), 127.05-135.32 (Ar-H), *Anal.* C17H16N2OCl2 (Cald.) C 60.90, H 4.81, N 8.35, O 4.77, Cl 21.15, Found C 60.85,H 4.76, N 8.27, O 4.68, Cl 21.05, IR, 3525 cm^{-1} (−CHOH), 1560 cm^{-1} (−CH=N)

A solution of ligand (1 mml) dissolved in dichloromethane was added dropwise in a Schlenk line flask containing degassed CH_2Cl_2 solution of $[RuCl_2(PPh_3)_3]$ (1 mmol). The reaction mixture was stirred for 30 minutes at room temperature which brings change in color from yellow to brown. The solution of reaction mixture was concentrated to 1 ml under reduced pressure and n-hexane was added to cause precipitation. The precipitate was collected and recrystallised in CH_2Cl_2-n-hexane and obtained in analytically pure form.

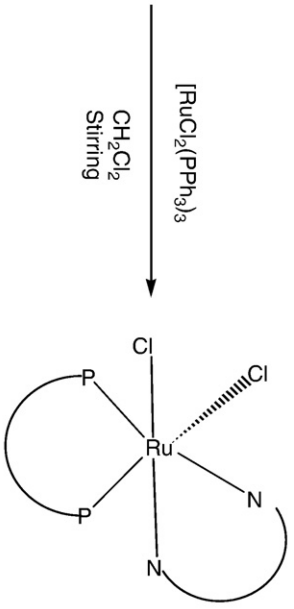
Yield 81%, color brown, mp 152 °C, ^1H NMR ($CDCl_3$): ∂ (ppm) 3.40-3.46 (m 4H -CH2), 3.89 (s 1H -CH), 4.47 (br 1H -CHOH), 8.84 (−CH=N), 7.25-7.37 (m, Ar-H), $^{31}P\{^1H\}$ NMR ($CDCl_3$): ∂ (ppm) 45.76, 4.25, *Anal* C53H46N2P2OCl4Ru (Cald.) C 61.70, H 4.49, N 2.71, O 1.55, P 6.00, Cl 13.74, Ru 9.79, (Found), C 61.65, H 4.40, N 2.67, O 1.51, P 5.96, Cl 13.68, Ru 9.75

Single crystal X-ray diffraction study was carried out using Oxford Diffraction X-calibur Eos Gemini diffractometer with graphite-monochromated Mo *Kα* radiation with the wavelength of 0.71073 Å. Data were analyzed with "CrysAlis PRO" software [18] and the collected data was reduced by using the "CrysAlis PRO" program. An empirical absorption correction using spherical harmonics was implemented in "SCALE3 ABSPACK" scaling algorithm. The crystal structure was solved by direct methods using SHELXS-97 [19] and the refinement was carried out against F^2 using SHELXL-97 [19]. All non-hydrogen atoms were refined anisotropically. The summary of crystallographic data and details of the structure refinement parameters are given in Table 1.

MTT assay was performed exactly as reported in literature [20]. Briefly, MDA-MB-231 and BxPC-3 cells were seeded at a density of $3x10^3$ cells per well in 96-well microtiter culture plates. After overnight incubation, cells were exposed to the indicated concentrations



1,3-bis{[(E)-(2-chlorophenyl)methylidene]amino}-2-propanol, L



Ru(II) complex

Scheme 1. Schematic representation of synthesis of Ru(II) complex.

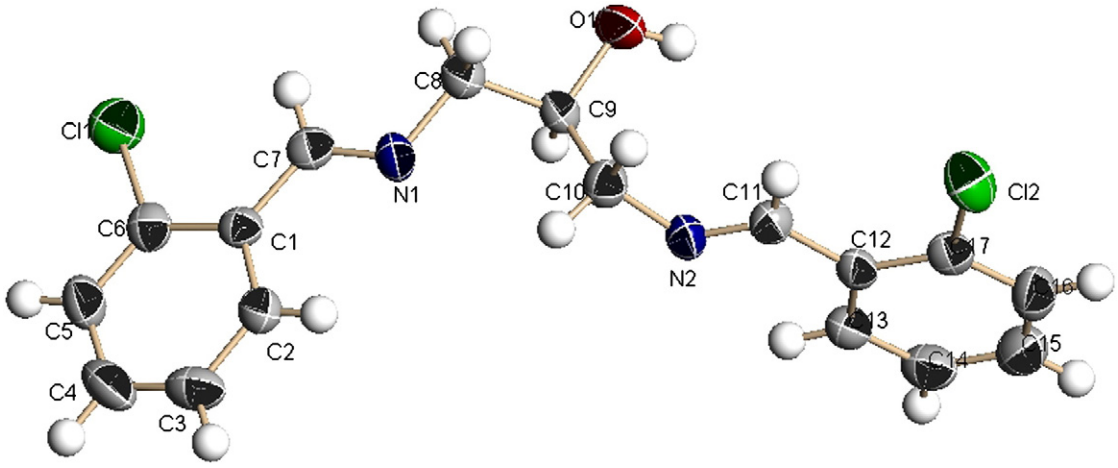


Fig. 1. ORTEP view of the ligand, L.

of ligand and Ru(II) complex for 96 h. Each treatment had eight replicate wells and the amount of DMSO in reaction mixture never exceeded 0.1%. Moreover, each experiment was repeated at least three times and a representative observation is reported.

The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in both MDA-MB-231 breast cancer and BxPC-3 pancreatic cancer cells treated with the ligand and its metal complex for 96 h, following the vendor's protocol [9]. The data presented here represents a mean of at least three repeats.

Results are expressed as mean ± SE of at least three independent observations. Student's *t*-test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. p-values<0.05 were considered statistically significant

Results and discussion. Ru(II)-Schiff base complex was synthesized by interaction of ligand, 1,3-bis{[(*E*)-(2-chlorophenyl)methylidene] amino}-2-propanol, L with $[RuCl_2(PPh_3)_3]$ in dichloromethane in schlenk line flask by stirring for 30 minutes resulting in to brown colored solution (Scheme 1).

The free ligand, 1,3-bis{[(*E*)-(2-chlorophenyl)methylidene]amino}-2-propanol, L obtained by the reaction of 2-chlorobenzaldehyde with 1,3-diaminopropanol, crystallizes in the non-cetrosymmetric orthorhombic *Pca*2_1 space group with all atoms located in general positions. The X-ray crystal structure shows 1,3-bis{[(*E*)-(2-chlorophenyl)methylidene]amino}-2-propanol in its asymmetric unit (Fig. 1), and there are four such molecules in the unit cell (Z = 4) and the relevant parameters are given in Tables 1–5. The observed C–C and C–N bond lengths as well as the bond angles of the molecule are in the normal range and are in good agreement with those observed for the corresponding compounds. The Cl–C bond lengths are in the range from 1.747(12) to 1.756(13) Å.

The free ligand, L and its Ru(II) complex is characterized by IR investigations in which the absorption band corresponding to OH-group appearing at 3525 cm^{-1} in the free ligand remains unchanged in the Ru(II) complex concluding that OH-group of ligand doesn't

Table 2
Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for eamu41. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
C(1)	−331(7)	7291(6)	3882(2)	37(1)
C(2)	−1907(8)	7867(7)	3649(2)	45(1)
C(3)	−3491(8)	8414(8)	3878(3)	57(2)
C(4)	−3540(10)	8404(9)	4348(3)	65(2)
C(5)	−2015(9)	7815(8)	4603(2)	57(2)
C(6)	−490(7)	7269(7)	4366(2)	44(1)
C(7)	1317(8)	6767(7)	3636(2)	42(1)
C(8)	3152(8)	6467(7)	2977(2)	48(1)
C(9)	3841(7)	7924(7)	2669(2)	39(1)
C(10)	2446(8)	8332(7)	2295(2)	45(1)
C(11)	3350(7)	9955(8)	1650(2)	43(1)
C(12)	3862(6)	11531(7)	1395(2)	38(1)
C(13)	3968(7)	13148(7)	1616(2)	47(1)
C(14)	4566(8)	14620(8)	1389(2)	56(2)
C(15)	5054(8)	14534(9)	924(2)	60(2)
C(16)	4964(8)	12965(9)	690(2)	57(2)
C(17)	4372(7)	11490(7)	932(2)	43(1)
Cl(1)	1397(2)	6559(2)	4696(1)	72(1)
Cl(2)	4290(2)	9540(2)	621(1)	62(1)
N(1)	1470(6)	7014(6)	3210(2)	45(1)
N(2)	2839(6)	9970(6)	2063(1)	41(1)
O(1)	5541(6)	7315(6)	2473(2)	60(1)

Table 3
Bond lengths [Å] and angles [°] for ligand, L.

C(1)-C(2)	1.400(8)
C(1)-C(6)	1.410(7)
C(1)-C(7)	1.449(7)
C(2)-C(3)	1.392(8)
C(2)-H(2)	0.9300
C(3)-C(4)	1.363(10)
C(3)-H(3)	0.9300
C(4)-C(5)	1.406(10)
C(4)-H(4)	0.9300
C(5)-C(6)	1.368(8)
C(5)-H(5)	0.9300
C(6)-Cl(1)	1.756(6)
C(7)-N(1)	1.255(7)
C(7)-H(7)	0.9300
C(8)-N(1)	1.457(7)
C(8)-C(9)	1.514(7)
C(8)-H(8A)	0.9700
C(8)-H(8B)	0.9700
C(9)-O(1)	1.436(6)
C(9)-C(10)	1.516(7)
C(9)-H(9)	0.9800
C(10)-N(2)	1.453(6)
C(10)-H(10A)	0.9700
C(10)-H(10B)	0.9700
C(11)-N(2)	1.253(6)
C(11)-C(12)	1.464(7)
C(11)-H(11)	0.9300
C(12)-C(17)	1.392(7)
C(12)-C(13)	1.397(7)
C(13)-C(14)	1.376(8)
C(13)-H(13)	0.9300

Symmetry transformations used to generate equivalent atoms:

participate in coordination whereas the absorption band corresponding to imine nitrogen of free ligand appearing at 1560 cm^{-1} is shifted to low-frequency ca. 1555 cm^{-1} indicating the coordination of imine nitrogen to Ru(II) ion. The Ru(II) complex shows stretching vibration at 3160 cm^{-1} and 255 cm^{-1} assigned to phosphine-CH and RuCl vibrations, respectively while the other bands were found at their expected positions.

The $^{31}P\{^{1}H\}$ NMR spectrum of Ru(II) complex shows a typical AX pattern, which correspond to two doublets observed at ca. ∂ 45.76 and ∂ 40.25 with $^{2}J_{P\text{-}P}$ 34.70 Hz suggesting that one of the P atoms is *trans* to imine nitrogen and second one is *trans* to chlorine atom (Fig. 2) [21]. This also indicates the magnetic nonequivalence of the two phosphorus atoms and thus, confirms that Ru(II) complex exists in *cis* geometry [22,23].

The ^{1}H NMR spectrum of free ligand, L exhibits a signal appearing at 8.79 ppm assigned to –CH = N proton while the chemical shifts for –CH2 (m 4H), -CH (s 1H), -CH-OH (br 1H) and aromatic protons appear at 3.14-4.28, 4.2, 4.46 and 7.25-7.36 ppm, respectively. The $^{13}C\{^{1}H\}$ NMR spectrum of ligand shows expected number resonance signals appearing at 65.0 ppm, 70.97 ppm, 159.89 ppm attributed to –CH2, -CH-OH and azomethine carbons, respectively while the resonance signals for aromatic carbons appear in 127.05-135.32 region (Figs. 3,4).

The ^{1}H NMR spectrum of Ru(II) complex shows a considerable high-frequency shift in resonances of proton and carbon with respect to the free ligand. The ^{1}H NMR spectrum of the Ru(II) complex shows a series of multiplets for phosphine phenyl groups and phenyl group in the 7.25-7.37 ppm region, corresponding to 26 hydrogen atoms while the resonance signals for CH_2, -CH, -CHOH and -CH = N appear at 3.40-3.46, 3.89, 4.47 and 8.84 ppm, respectively (Fig. 5).

The electronic spectrum of Ru(II) complex measured in dichloromethane shows presence of bands at 212, 245, 272 and 322 nm. The bands below 300 nm is attributed to intraligand π-π* transitions while the band at 322 nm is assigned to metal-to-ligand charge transfer (MLCT) transition [24].

The effect of ligand and its Ru(II) complex was observed on the proliferative potential of human breast cancer cells MDA-MB-231 and pancreatic cancer cells BxPC-3. The antiproliferative effect of the compounds was evaluated by measuring the level of cell proliferation after incubation of cells with the test samples, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay, which evaluates the capacity of the mitochondrial enzyme succinate dehydrogenase of viable cells to reduce MTT to formazan crystal [25]. The results, expressed as percentage of cell proliferation compared with cells control (Cells treated with vehicle, DMSO 0.1%) as presented in Fig. 6. The Ru(II) complex caused an efficient dose-dependent inhibition of the proliferation of both cancer lines (Fig. 6A and B). The results show that MDA-MB-231and BxPC-3 cells were generally less sensitive to ligand which was only able to inhibit 50% or less of cell proliferation at 50 μM. However, both the cancer lines were more susceptible to Ru(II) complex demonstrating a growth inhibition of > 95% in both the cases. The inhibition of overall cell growth of cancer cells by anticancer agents is known to be accompanied by induction of apoptosis [26]. Therefore, in order to examine the relationship between cell proliferation inhibition induced by the complex with apoptosis induction, we tested the ability of the complex to cause apoptosis in human breast and pancreatic cancer cell lines. As shown in Fig. 7A & B, histone/DNA ELISA assay of apoptosis detection revealed a dose dependent increase in apoptotic cell death induced by Ru(II) complex in both the cell lines tested. Thus Ru(II) complex presented in the current study provide worthwhile potential as an anticancer agent.

Table 4
Anisotropic displacement parameters ($Å^2 x\ 10^3$)for ligand, L. The anisotropic displacement factor exponent takes the form: $-2\pi^2[\ h^2a^{*2}U^{11} + \ldots + 2\ h\ k\ a^*\ b^*\ U^{12}]$.

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
C(1)	39(3)	31(3)	42(3)	−1(2)	0(2)	0(2)
C(2)	46(3)	43(3)	46(3)	5(3)	5(3)	0(3)
C(3)	39(3)	52(4)	80(5)	−1(3)	−3(3)	7(3)
C(4)	58(4)	57(4)	80(5)	−7(4)	28(4)	6(3)
C(5)	69(4)	57(3)	45(4)	−1(3)	18(3)	3(3)
C(6)	48(3)	41(3)	44(3)	−1(3)	1(3)	−5(3)
C(7)	44(3)	35(3)	46(3)	0(3)	−3(2)	0(2)
C(8)	51(3)	43(3)	50(3)	5(3)	8(3)	3(3)
C(9)	38(3)	42(3)	37(3)	−2(2)	6(2)	2(2)
C(10)	43(3)	51(3)	41(3)	3(3)	−1(2)	−2(3)
C(11)	41(3)	47(3)	40(3)	4(3)	−1(2)	3(2)
C(12)	28(2)	49(3)	38(3)	5(2)	1(2)	3(2)
C(13)	46(3)	50(3)	45(3)	1(3)	4(3)	8(3)
C(14)	44(3)	51(3)	71(4)	0(3)	1(3)	5(3)
C(15)	48(3)	62(4)	70(4)	17(4)	2(3)	1(3)
C(16)	50(3)	76(4)	44(4)	14(3)	0(3)	0(3)
C(17)	31(3)	57(3)	40(3)	2(3)	−7(2)	4(2)
Cl(1)	76(1)	92(1)	50(1)	3(1)	−6(1)	23(1)
Cl(2)	64(1)	74(1)	48(1)	−14(1)	5(1)	−5(1)
N(1)	43(3)	55(3)	37(3)	5(2)	8(2)	1(2)
N(2)	36(2)	48(2)	39(2)	5(2)	5(2)	0(2)
O(1)	39(2)	59(3)	81(3)	17(2)	16(2)	8(2)

Table 5
Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2 x\ 10^3$) for ligand, L.

	x	y	z	U(eq)
H(2)	−1895	7886	3329	54
H(3)	−4515	8785	3712	69
H(4)	−4591	8791	4500	78
H(5)	−2043	7796	4923	69
H(7)	2278	6239	3796	50
H(8A)	2907	5434	2793	58
H(8B)	4089	6176	3202	58
H(9)	4071	8972	2854	47
H(10A)	2446	7393	2071	54
H(10B)	1225	8390	2431	54
H(11)	3406	8887	1499	51
H(13)	3625	13231	1924	56
H(14)	4646	15677	1546	67
H(15)	5441	15537	771	72
H(16)	5288	12892	381	68
H(1)	6150(80)	8210(80)	2320(20)	56(18)

Conclusion. We have successfully isolated a novel Ru(II)-Schiff base complex derived from 1,3-bis{[(*E*)-(2-chlorophenyl)methylidene] amino}-2-propanol. The synthesized compounds were characterized

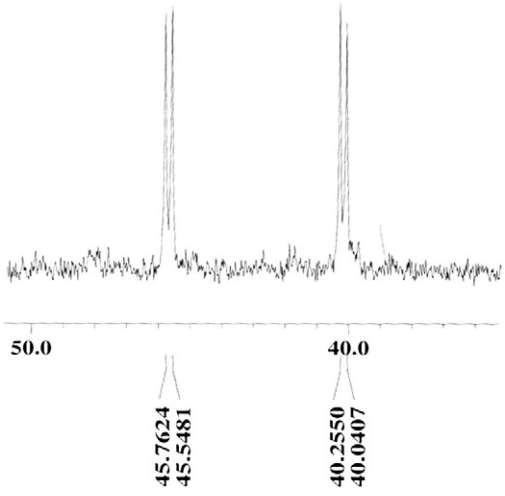


Fig. 2. $^{31}P\{^{1}H\}$ NMR spectrum of Ru(II) complex.

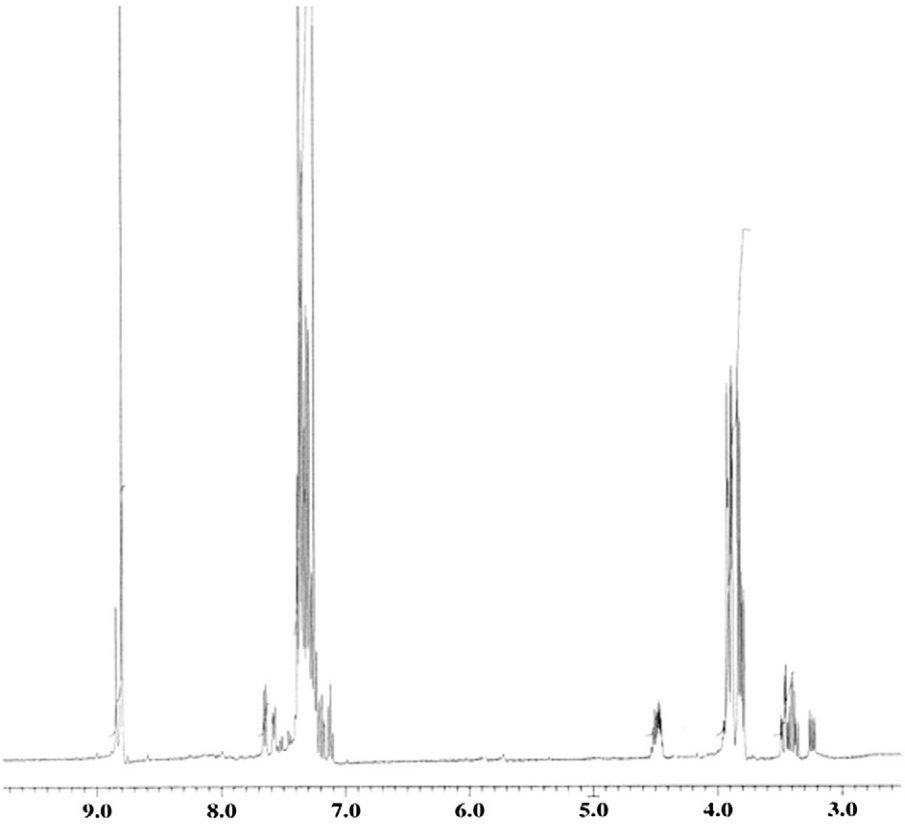


Fig. 3. ^{1}H NMR spectrum of ligand.

on the basis of various spectroscopic studies and X-ray crystallography in case of ligand. The *in vitro* antitumor activity was examined by screening their ability to inhibit the cancer growth in various human cancer cell lines, (MDA-MB-231) and (BxPC-3). The studies demonstrate that Ru(II) complex show excellent *in vitro* antiproliferative and apoptosis inducing activity.

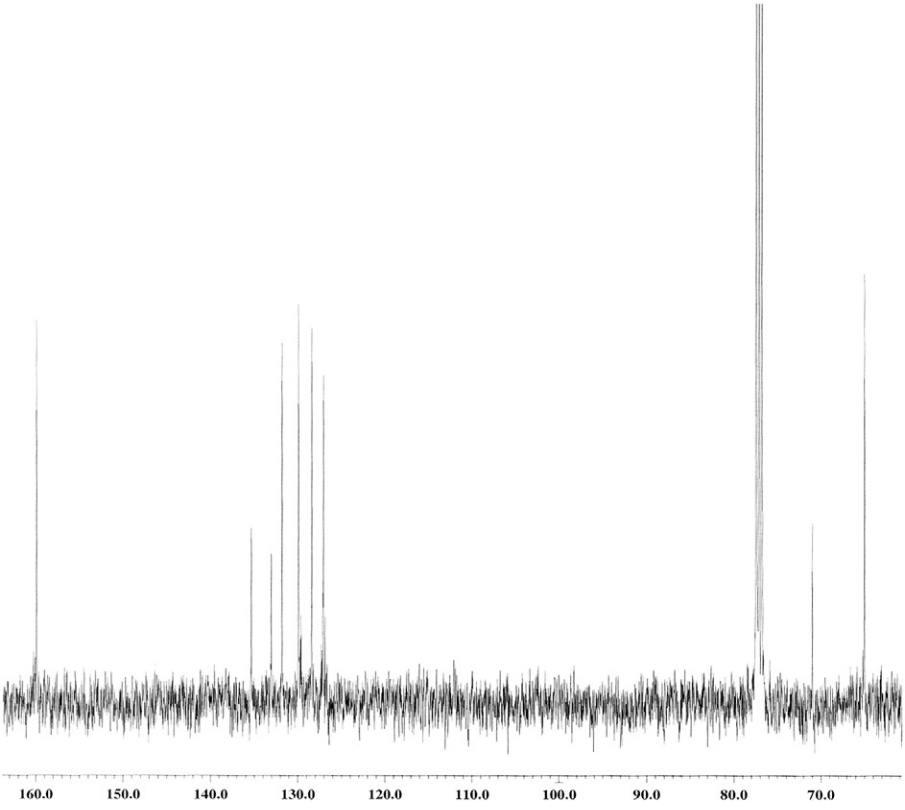


Fig. 4. $^{13}C\{^{1}H\}$ NMR spectrum of Ligand.

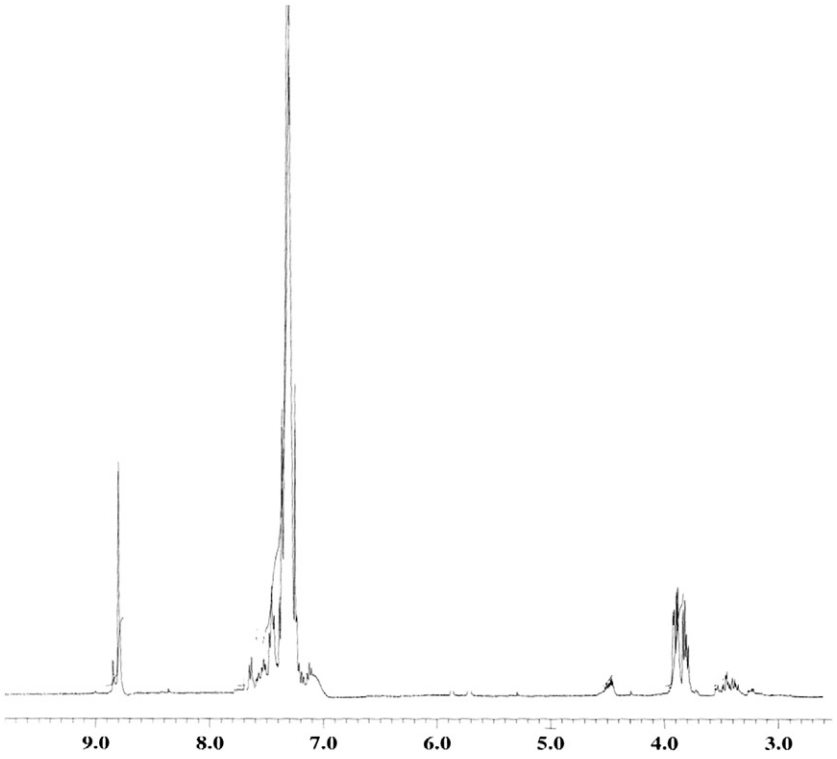


Fig. 5. ^{1}H NMR spectrum of Ru(II) complex.

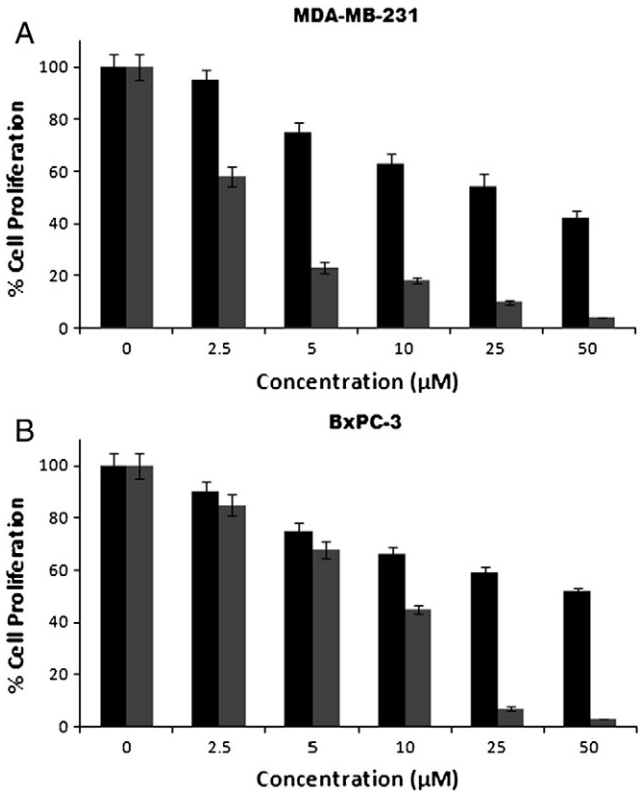


Fig. 6. Effect of ligand (■) and its Ru complex (■) on cell growth of **(A)** breast cancer cells (MDA-MB-231) and **(B)** pancreatic cancer cells (BxPC-3) as detected by MTT assay after 4 days of treatment. Cells were incubated with increasing concentrations of the test agents, as indicated in the figure. All results are expressed as percentage of control (± SE). Values reported are statistically significant with $p<0.05$ as compared to control (untreated cells) and also Ru complex compared to the activity of its parent compound.

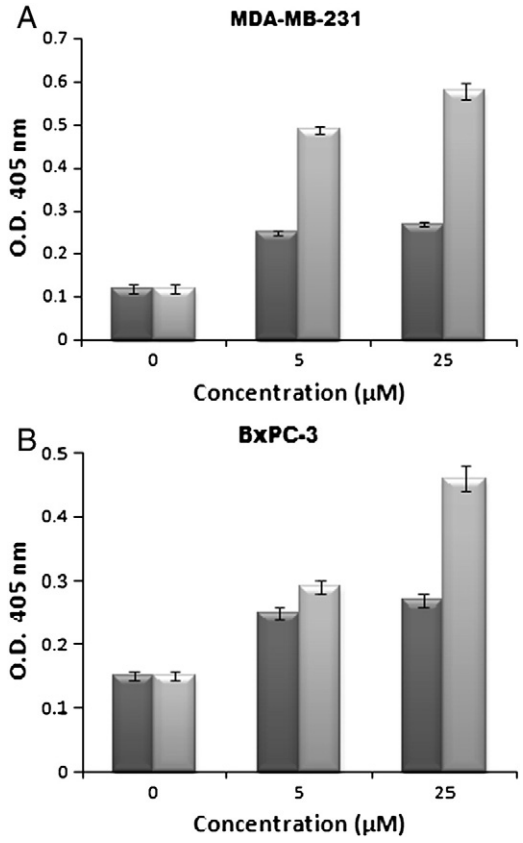


Fig. 7. Effect of ligand (■) and its Ru complex (■) on apoptosis induction in **(A)** breast cancer cell line MDA-MB- 231 and **(B)** pancreatic cancer cell line BxPC-3. Values reported are statistically significant with $p<0.05$ as compared to control (untreated cells) and also Ru complex compared to the activity of its parent compound.

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Appendix A. Supplementary material

CCDC 821262 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk./data_request/cif. Supplementary materials related to this article can be found online at doi:10.1016/j.inoche.2012.03.019.

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