International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

Research Article **CODEN: IJPNL6**

Cytotoxicity Assay of Nickel and Cobalt (II) Complexes of 5-(4-Nitro Phenyl)-4-Amino-3-Mercapto Propenyl-1,2,4-Triazole on HepG2 Cell Line

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ABSTRACT

Two new complexes with formula $[M(NMP.TRZ)(H_2O)_3](NO_3)_2.3EtOH$ (where M is Ni and Co(II) ions respectively, NMP.TRZ is (5-(4-Nitro Phenyl)-4-Amino-3-Mercapto Propenyl-1,2,4-Triazole) have been synthesized by conventional method. These complexes have been characterized by spectroscopic methods such as (ultraviolet- visible and infrared), as well as to thermal gravimetric, metal analyses, microanalyses, conductivity, magnetic moment and molar ratio method. To measure the biologic activity and potential anticancer efficacy of these compounds, they have been compared with cisplatin on human hepatocarcinoma HepG2 cell lines in different eight concentrations (2000, 1000, 500, 250, 125, 62.5, 31.25 and 15.625 μg/ml) respectively, in the time of exposure 72 hrs. The results exhibit that the three prepared complexes i.e. ligand (NMP.TRZ) and its metal complexes have shown higher ratios cytotoxicities compared to cisplatin against HepG2 cell lines in most selected concentrations. Based on the obtained results of biological test, these compounds may be potentially being considered as good anticancer candidates for further pharmacological studies.

Keywords: complexes, 3-mercapto propenyl, anticancer, cisplatin, HepG2.

INTRODUCTION

Bioinorganic chemistry is a rapidly developing field dealing with the synthesis and biological investigation of inorganic complexes **[1,2]**. In inorganic complexes, the central metal ion has a significant role in the development of anticancer metallodrug**.** Discovery of cytotoxic activity of cisplatin and its analogue such as carboplatin has led to an extensive search of new biologically active metal drugs **[3]**. Currently, cis-diamminedichloroplatinum (II) (cisplatin) is being used as an anticancer drug against several human cancers, such as: testicular cancer, Ovarian and bladder cancer, osteogenic Sarcoma, head and neck cancer, endometrial and cervical and non-small cell lung cancer **[4]**. Due to the frequent resistant to this drug during cancer chemotherapy **[5]**, as well as dose limiting toxic side effects of platinum anticancer drugs, there have been many attempts to find complexes with greater potency and less toxicity than the exiting clinical drug **[4,5]**. The chemistry and biological study of heterocyclic compounds has been an interesting field for a long time in medicinal chemistry. A number of heterocyclic derivatives containing nitrogen and sulphur atom serve as a unique and versatile scaffolds for experimental drug design **[6]**. In this paper, we report on the synthesis and structural characterization of nickel and cobalt (II) complexes derived from 5-(4-nitrophenyl) 4-amino-3-mercaptopropenyl-1,2,4-triazole (NMP. TRZ). Moreover, the *in vitro* cytotoxic assay on human liver carcinoma cells, HepG2 of the ligand and complexes was reported herein.

EXPERIMENTAL

Instruments: All the chemicals used in this work were of analytical reagent grade. FTIR spectra (KBr & CsI discs) were recorded in the Nujol media 4000-400 and 4000-200 cm^{-1} region on an IR Prestige-21 Spectrophotometer and electronic spectra of solids compounds dissolve in DMF solvent were recorded on a Shimadzu UV-160 Spectrophotometer. The elemental analysis was carried out using an EM-017 analyzer. Metal contents were estimated using an AA-680 Shimadzu Atomic Absorption Spectrophotometer. Magnetic susceptibilities were measured on a Faraday balance (Burker BM6 instrument) at room temperature. The electrical conductance measurements were recorded using 10^{-3} molar solutions in DMF with a WTW conduct meter type. TGA experiments were performed on Perkin-Elmer model 4000 instrument. Melting point apparatus of Gallencamp M.F.B-60 was used to measure melting point of all prepared compounds. The cells used in cytotoxicity evaluations were cultivated in a CO_2 incubator (SANYO, incubator Japan) and the evaluations were carried out with a micro ELISA reader ASYS, at a wavelength of 492 nm.

Synthesis of 5-(4-nitrophenyl) 4-amino-3-mercapto propenyl-1,2,4-triazole (NMP.TRZ) as a ligand : A suspension of (0.1 mol) of potassium 3-(4-nitrobenzoyl) dithiocarbazate was prepared according to reference method **[7]** in absolute alcohol, (0.2 mol) of hydrazide hydrate and 6 ml of water were refluxed for 3 hrs. The colour of the reaction mixture changed to green with the evolution of hydrogen sulfide gas and a homogenous solution resulted. Cold distilled water (100 mL) was added and the solution was acidified with conc. HCl. The precipitate solid was filtered, washed with 2x30 mL portions of cold water, and recrystallized. To a stirred solution of previously compound i.e. 5-(4-nitrophenyl) 4-amino-3-mercapto-1,2,4-triazole (0.003 mol) in 15 mL absolute ethanol was added slowly (0.003 mol) KOH. To the stirred reaction mixture the appropriate allyl bromide (0.003 mol) was added drop wise and the mixture was refluxed for 1 hr, after cooling, filtered and the filtrate was poured into ice-cold water (500 mL) the crude product was recrystallized from ethanol **(Fig 1)**. (Yield 63.00%, m.p= 176-178 ^oC, M.Wt=277.00 $gm.mol^{-1}$, analysis (found): C=46.71, H=3.55, N=25.01 and S=10.11, (calculated): C=47.65, H=3.97, N=25.27 and S=11.02.

Synthesis of Ni and Co(II) complexes: This complexes was obtained by heating under reflux a 1:1 molar mixture of $Ni(NO_3)_2.6H_2O$ for Ni(II) complex and $Co(NO_3)_2.6H_2O$ for Co(II) complex (1mmole) and 5-(4-nitrophenyl) 4-amino-3-mercapto propenyl-1,2,4-triazole (NMP.TRZ) (0.277gm, 1mmole) in (15 mL) of ethanol for 15 Min. A clear orange and brown transparent solution respectively, was separated by filtration and kept in Petri dishes. After a few days, orange and brown crystals were collected. The physical properties for $C_{17}H_{35}N_7O_{14}SNi$ $[Ni(NMP.TRZ)(H_2O)_3](NO_3)_2.3EtOH$ complex is: yield 78.94%, m.p= 101 ^oC (dec.), M.Wt=651.69 $gm.mol^{-1}$, analysis (found): C=30.16, H=4.30, N=14.51, S=4.14 and Ni=9.12, (calculated): C=31.30, H=5.06, N=15.03, S=4.91 and Ni=9.00. While for $C_{17}H_{35}N_7O_{14}SCo$ $[Co(NMP.TRZ)(H_2O)_3](NO_3)_2$ .3EtOH complex is: yield 76.19%, m.p= 78 ^oC (dec.), M.Wt=651.93 $gm.mol^{-1}$, analysis (found): C=30.51, H=4.55, N=14.63, S=4.08 and Ni=8.10, (calculated): C=31.29, H=5.36, N=15.03, S=4.90 and Co=9.00.

Formation of complexes in solution state: Formation of both metal complexes of nickel and cobalt have been studied in solution state using ethanol as a solvent, in order to determine [M/L] ratio using molar ratio method **[8]**. A series of solutions have been prepared having a constant concentration 10^{-3} M for each metal ion and ligand. The ratio have been determined from the relationship between the absorption of the absorbed light and molar ratio of (M:L) at λ_{max} of maximum absorption.

In vitro cytotoxic testing: Cell lines used for testing *in vitro* cytotoxicity included HepG2 derived from Human hepatocellular liver carcinoma cell lines were supplied from the Biotechnology Research Center Al-Nahrain University. The cells were cultured in controlled environment in modified DMEM medium supplemented with 10% fetal calf serum, along with 1% non-essential amino acids with L-glutamine, 0.2% sodium bicarbonate, 1% sodium pyruvate and 1% of antibiotic mixture (10000 units penicillin and 10 mg streptomycin per mL) in 25 cm^2 culture flask at 37 ^oC in an incubator with humidified atmosphere and 5% carbon dioxide. Cells were kept in *log* phase by supplementation with fresh medium after removal of cell suspension aliquots, two or three times weekly. The cells were washed and resuspended in the above medium and 100 µL of this suspension was seeded in 96 well flat bottom plates **[9]**. After 24 hrs incubation, the cells were treated for 3 days with test compounds at concentrations ranging from 15.625 to 2000 µg/ml were freshly prepared in DMSO and diluted with DMEM in order to obtain the desired final concentrations. Less than 1% of the solvent was available at the final dilutions obtained. All of the procedures concerning the cell culture

maintenance, drug dissolution, and treatment were carried out in a "*Napaco*" Laminar flow cabinet.

Cell viability determination (neutral red assay): After elapsing the selected incubation period (72 hrs) of examined compounds as well as control positive and control negative, 50 µl/well of neutral red dye freshly prepared were added to each well and incubated again for 2 hrs, viable cells will uptake the dye and the dead cells will not. The plates washed with Phosphate Buffer Saline (PBS) to remove the excess dye, then 100µl/well of eluent solution were added to each well to draw out the dye from the viable cells. Optical density of each well was measured by using ELISA reader at a transmitting wave length on 492nm **[10], (Fig 3)**.

Statistics: The data processing included the variance *ANOVA* test with $P \square 0.05$ taken as significance level, using Microsoft EXCEL for PC, **(Table 4)**.

RESULTS AND DISCUSSION

Characteristic spectra of ligand and its metal complexes: Two complexes with formula [Ni(NMP.TRZ) $(H_2O)_3$] $(NO_3)_2$.3EtOH and [Co(NMP.TRZ) $(H_2O)_3$]$(NO_3)_2$.3EtOH were prepared by interaction between metal salts of Ni and Co with new ligand (NMP.TRZ) in molar ratio 1:1. The molar conductance values of these complexes in DMF refer to electrolyte behavior of these new complexes **(Table 1)**. The chemical analysis and molar conductance data support the above suggested formulation of these complexes. In the electronic absorption spectra of new ligand exhibited three main bands at 260, 271 and 355 nm respectively. The one band due to intra ligand ($\pi\rightarrow\pi^*$) transition located on the –C=C– group, the second absorption band arise from ($n\rightarrow\pi^*$) transition within the –C=N– group, while the third band also attributed to ($n\rightarrow\pi^*$) transition but located on the nitrogen atom of the imine group **[11]**. The magnetic moment of Ni (II) complex is 3.05 B.M. which suggest octahedral geometry of this complex. The Ni(II) complex show the presences three bands attributed to d-d transition in the region 10177, 17931 and 26422 cm^{-1} which were assigned to $^3A_2g\rightarrow{}^3T_2g(\upsilon_1)$, $^3A_2g\rightarrow{}^3T_{1g(F)}(\upsilon_2)$ and $^3A_2g\rightarrow{}^3T_{1g(p)}$ respectively **[12]**, as well as another two bands appeared at 14814 and 29368 cm^{-1} indicate to spin-forbidden transition $^3A_2g\rightarrow{}^1Eg$ **[13]** and charge transfer from L→M respectively. The value of the magnetic measurement (4.52) B.M indicates that the brown Co(II) complex to be paramagnetic and is characteristic of high spin cobalt ion in octahedral geometry species. The spectrum of this complex showed two bands at 19230 and 25062 cm^{-1} which attributed to ν_2 and ν_3 respectively**[14]**, the value of first transition was calculated theoretically and found to be 10864 cm^{-1}. The different ligand filed parameters have been calculated using Tanaba-Sugano diagram for d^7 system, the results are found in **(Table 1)**. In the FTIR spectrum of ligand exhibit essential bands due to olefin and vinylic groups at 1558 and 864 cm^{-1}**[15]** respectively. These bands undergoes shifting to higher frequencies in both complexes of olefin group C=C, also the stretching frequency related to ν(CH) olefin was shifted to higher frequencies, while the bending band of $\delta=CH_2$ group shifted to lower frequencies in both complexes this indicated that the ligand is coordinated with metals by Pi bond, this coordination was supported by appearance of ν(M-C) band in the region (420 and 428 cm^{-1}) **[11,16]**. The bands appeared at 3441 and 3360 cm^{-1} region due to the stretching frequency of asymmetric and symmetric NH_2 group which disappearance in both complexes, while band of stretching mode of N-N band is remained without change in both complexes at 1014 cm^{-1}, another band appeared at 752 cm^{-1} which attributed to ν(C-S) band which undergoes a shifting to lower frequencies in all complexes, which support coordination of ligand with metal ions by these group i.e. NH_2 and C-S in addition to pi bond, the result reflect the tridentate behavior of this new ligand with both metal complexes, more evidence new bands appeared due to the stretching frequency of (M-S) and (M-N) bond **[11,16] (Table 2)**.

Thermal gravimetric analyses (TGA): The thermogram of solid complexes showed that the weight loss was measured from 40 to 900 ^{o} C for nickel complex and from 40 to 860 ^{o}C for cobalt complex at 20.00 ^{o} C /min. In both complexes the decomposition occurred by six steps. The first stage corresponding to loss of three coordination water molecules from these complexes, the second stage refer to loss of three uncoordination ethanol molecules in both complexes, the third stage indicated to decompose two nitro molecules in Ni and Co (II) complexes. Another two stages exhibited in the TGA charts due to decomposition of ligand (NMP.TRZ) by two steps the first of propenyl group and the second for the remained atoms of ligand except sulfur atom i.e. $C_8H_6N_5O_2$ in both complexes, the six step which observed refer to decomposition of metals with sulfur atom of ligand and it is the final pyrolysis product **(Table 3)**.

Solution study: Molar ratio method support that the ratio of metal to ligand is 1:1 for both complexes.

Suggested structure of new complexes: Suggested structures of prepared complexes are shown in **Fig 2**.

Cytotoxic assay: We have examined the cytotoxicity effects of ligand and its metal complexes (**Figs 1, 2**) on human liver cell lines as potential candidates for human cancer therapy and the results shown in **(Table 4)**. In this table, colonogenic results (mean ± SD) are listed as the percentage of colonies which inhibition after exposure to different concentrations of prepared compounds in comparison with control positive. The rank order of cisplatin cytotoxicity on selected cell lines is in well agreement with the previous publication and the cell lines sensitivities to this anticancer drug. This may be counted as a proof for the accuracy of cytotoxicity assay. The free ligand (NMP.TRZ) exhibit cytotoxicity against HepG2 cell lines with inhibition percentage less than cis-Pt and its metal complexes in all concentrations, and this effect may be due to presence of imine group, olefin and vinylic groups as well as sulphur atom and triazole ring which own the ability to inhibit the proliferation of cell lines and this explain the effect of this new ligand on this cell lines. The statistical data of Ni (II) complex recorded highly inhibition rates among the prepared compounds in all concentrations even highly than cis-Pt itself and the higher inhibition rate observed at 62.5 µg/ml concentration without any significant difference with other concentrations (250 and 125) and the results recorded with significant difference P □ 0.05 with another compounds i.e. control positive, (NMP.TRZ) ligand, and Co(II) complex in all selected concentrations **(Fig 3)**, while the Co(II) complex have potent as anticancer activities against human hepatoma cell lines in time of exposure 72 hrs but less than the control positive and Ni(II) complex and a slight higher than (NMP.TRZ) ligand in most concentrations. From the results presented in this work, many factors may be responsible in the activity of these complexes in pharmacological field like size of metal, charge distribution, geometry shape, ionic character, and polarity **[17]**. For example, the results showed the nickel (II) complex have the higher cytotoxicity this may be due to high polarity and large size of this metal in this complex than Co (II) ion. Since the high polarity gives covalent character in ionic bond to the complexes and made easy to enter in the cell. From the above data, we found cytotoxic, cytostatic, and antiproliferative effect of our prepared compounds (*in vitro*) at different concentrations and this effect may be attributed to blocking of DNA or RNA synthesis or blocking the protein synthesis of the cells, another suggested idea is that it led the cells to the apoptotic pathway so the inhibition rate is found high with some concentrations. Also some compounds have low cytotoxic effect in high concentrations than other concentrations i.e. low and middle concentrations, this is common in toxicology and called Hormesis which means the compound showed inhibition effect at low concentrations but not at high concentrations **[18]**.

Table 1: Electronic spectra, conductance in DMF solvent and magnetic moment (B.M) for the prepared ligand and its metal complexes

Comp.	Absorption Bands (cm^{-1})	Assignment	B°	B′	B	Dq/B′	10Dq	15B′	μ_{eff} B.M.	μsm^{-1}	Suggested geometry
L	28164	n→π*	---	---	---	---	---	---	---	---	---
	36900	π→π*									
	38461	π→π*									
NiL	14814	$^3A_2g \rightarrow {}^1Eg$	1035	674.01	0.65	1.21	10177	19348	3.05	109.0	O.h
	10177(cal.)	$^3A_2g \rightarrow {}^3T_2g$									
	17931	$^3A_2g \rightarrow {}^3T_1g_{(F)}$									
	26422	$^3A_2g \rightarrow {}^3T_1g_{(P)}$									
	29368	L→ NiCT									
CoL	10864(cal.)	$^4T_1g \rightarrow {}^4T_2g$	971	835.74	0.86	1.3	10864	11698.14	4.52	158	O.h
	19230	$^4T_1g \rightarrow {}^4A_2g$									
	25062	$^4T_1g \rightarrow {}^4T_1g_{(F)}$									

Where: CT→ Charge transfer

Table 2: Main band in FTIR of the ligand and its metal complexes in (cm^{-1})

Comp.	νNH_2	ν(C=C)	ν(CH) olefin	δ=CH_2	ν(C-S)	νN-N	ν(M-C)	ν(M-N)	ν(M-S)	Others
L	3441 m 3360 w	1558 sh	3035m	864 w	752 m	1014 w	---	---	---	---
NiL	---	1581 w	3062 w	802 w	694 w	1014 w	420 m	486 w	468 w	νEtOH= 3550 δH_2O= 887 w νM-O= 540 w νNO_{3-} 1357, 1234, 1200
CoL	---	1570 w	3062 w	829 w	694 w	1014 w	428 m	489 s	462 w	νEtOH=3550 δH_2O= 829 w νNO_3= 1365, 1234, 1200

Where: w=weak, s=strong, m=medium, sh=sharp

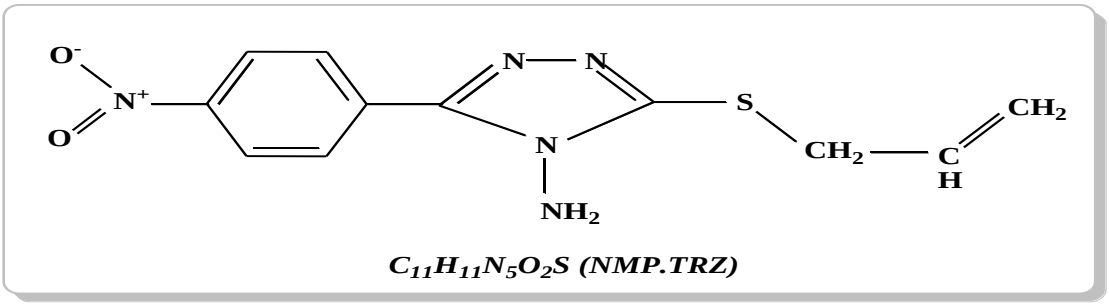
Table 3: Thermal analyses for Ni and Co (II) complexes

Comp.	Dissociation stages	Temp range in TG °C	Weight loss Found (Calc.)%	Decomposition assignment.
NiL	Stage I	40-105	8.40 (8.28)	Coordination water molecules.
	Stage II	105-250	21.00 (21.17)	Outer sphere EtOH.
	Stage III	250-310	19.23 (19.02)	$2NO_3$
	Stage IV	310-420	6.43 (6.29)	Propenyl group C_3H_5 of ligand.
	Stage V	420-850	31.52 (31.30)	$C_8H_6N_5O_2$ of ligand.
	Stage VI	850-900	13.66 (13.91)	NiS
CoL	Stage I	40-110	8.36 (8.28)	Coordination water molecules.
	Stage II	110-250	21.02 (21.16)	Outer sphere EtOH.
	Stage III	250-310	19.30 (19.02)	$2NO_3$
	Stage IV	310-420	6.49 (6.28)	Propenyl group C_3H_5 of ligand.
	Stage V	420-800	31.61 (31.29)	$C_8H_6N_5O_2$ of ligand.
	Stage VI	800-860	13.75 (13.94)	CoS

Table (4): Statistical data of (NMP.TRZ) and it metal complex as well as and control positive on HepG2 cells lines in time of exposure 72 hrs

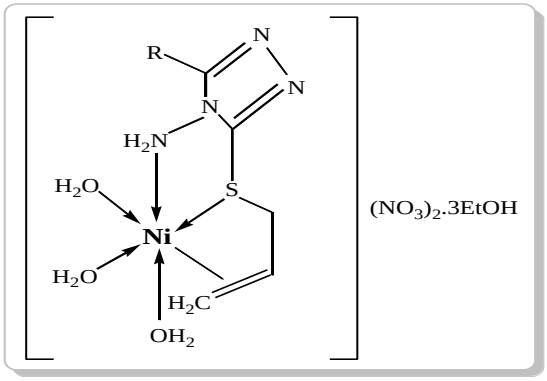
Treatment / ***Concentrations µg/ml***	***Inhibition rate%(means ±standard deviation SD)***			
	cis-Pt Control positive	**NMP.TRZ Ligand**	**Ni(II) Complex**	**Co(II) Complex**
2000	70.248 ± 0.626 A, a	53.369 ±0.021 B, a	71.787 ± 0.811 A, a	63.728 ± 0.820 C, a
1000	73.764± 0.733 A, b	55.795± 0.170 B, b	78.256± 0.654 C, b	65.705± 0.431 D, b
500	75.022 ± 0.031 A, c	62.803 ± 0.042 B, c	80.670 ± 0.321 C, c	67.385 ± 0.789 D, c
250	75.202 ± 0.129 A, c	59.177 ± 0.031 B, d	82.839 ± 0.012 C, d	65.139 ± 0.335 D, b
125	77.890 ± 0.836 A, d	57.771 ± 0.221 B, e	82.030 ± 0.467 C, d	68.104 ± 0.921 D, c
62.5	67.115 ± 0.701 A, e	51.267 ± 0.057 B, f	83.018 ± 0.022 C, d	68.373 ± 0.776 A, c
31.25	66.037 ± 0.501 A, e	40.521 ± 0.121 B, g	56.693 ± 0.833 C, e	52.021 ± 0.321 D, e
15.625	52.740 ± 0.282 A, f	21.743 ± 0.944 B, h	67.924 ± 0.956 C, f	41.285 ± 0.019 D, f

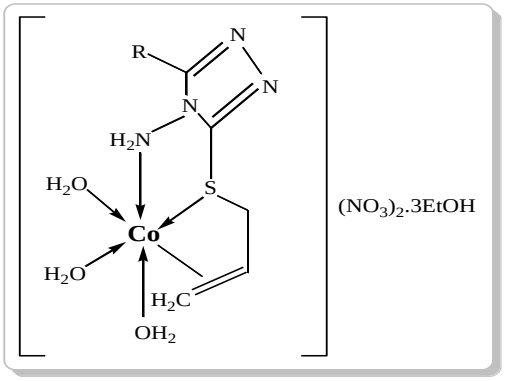
Differences A,B,C,D are significant (P<0.05) to compression row
Differences a, b, c, d, e, f, g, h are significant (P<0.05) to compression column



$C_{11}H_{11}N_5O_2S$ (NMP.TRZ)

Fig 1: Show the structure of 5-(4-nitrophenyl) 4-amino-3-mercapto propenyl-1,2,4-triazole (NMP.TRZ).





Molecular formula: $C_{17}H_{35}N_7O_{14}SNi$ ***Molecular formula: $C_{17}H_{35}N_7O_{14}SCo$***

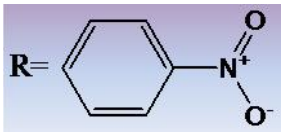
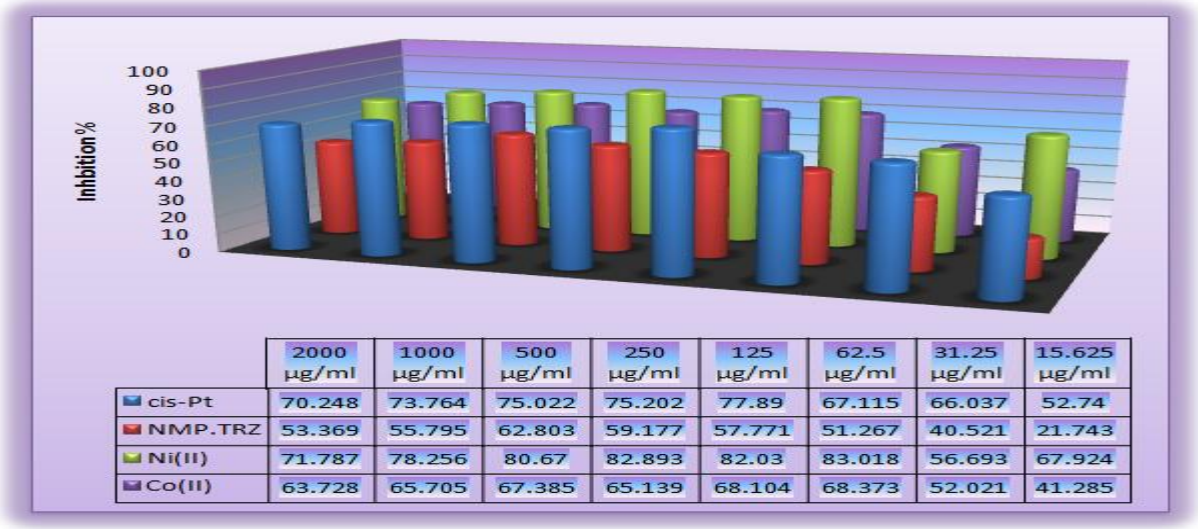
Where R= 

Fig 2 Suggested structure of the new prepared complexes.



	2000 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	62.5 µg/ml	31.25 µg/ml	15.625 µg/ml
cis-Pt	70.248	73.764	75.022	75.202	77.89	67.115	66.037	52.74
NMP.TRZ	53.369	55.795	62.803	59.177	57.771	51.267	40.521	21.743
Ni(II)	71.787	78.256	80.67	82.893	82.03	83.018	56.693	67.924
Co(II)	63.728	65.705	67.385	65.139	68.104	68.373	52.021	41.285

Fig (3): Shows the percentage inhibition rate in HpeG2 cell lines after exposure to (NMP.TRZ) and its metal complexes in time of exposure 72hrs comparable with control positive (cis-Pt).

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