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Enhancement of Anticancer Activity of N(4)1-(2-Pyridyl)piperazinyl 5-Nitroisatin Thiosemicarbazone on Chelation with Copper(II)

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5-Nitroisatin-4-(1-(2-pyridyl)piperazinyl)-3-thiosemicarbazone (Nitistpyrdlpz) and its Cu(II) complex were synthesized and characterized by CHN and thermal analysis and spectroscopic measurements *viz.* UV-vis, FTIR, ^{1}H NMR, ^{13}C NMR, ESI-HRMS, PXRD and EPR. In the complex, copper(II) ion is coordinated by terdentate thiosemicarbazone anion and one chloride ion in a distorted square planar geometry. The synthesized compounds against breast cancer cell lines; MCF-7 and MDA-MB-231 and epidermoid carcinoma; A431 showed that the complex contributed to reduce the percentage of cell viability toward all the tested cell lines but remarkable contribution toward MDA-MB-231 cell line. The IC_{50} of the complex and free ligand was found in the range of IC_{50} 0.85-1.24 μM and IC_{50} 3.28-3.53 μM, respectively. Among those cell lines, the complex may be the better anticancer agent toward MDA-MB-231 because of its action at micromolar concentration (IC_{50} 0.85 μM).

Keywords: Anticancer activity, Copper(II) complex, MDA-MB-231 cell line, 5-Nitroisatin, Thiosemicarbazone.

INTRODUCTION

Isatin, also known as tribulin or 1*H*-indole-2,3-dione and its derivatives such as tryptanthrin, 2-oxindoles, indirubins, sunitinib V, isatin thiosemicarbazone, *etc*. are important class of biologically active heterocyclic compounds that exhibit anticancer activity and anticancer target (tyrosine kinase, carbonic anhydrase, histone deacetylase and tubulin polymerization), antiviral activity (pox virus, SARS virus, Moloney leukemia virus, rhinovirus and vaccinia), antibacterial, anticonvulsant, antituberculosis, antidiabetic activities, *etc.* [1]. Recent studies have shown that substitution of isatin moiety at C-5 position by $–CH_3$, -F, -Cl, -Br, $-NO_2$, $-OCH_3$, $-OCF_3$ *etc*. produces the most active anticancer thiosemicarbazones. Those 5-substituted isatin thiosemicarbazone, N(4) substitution with hydroxamic acid exhibited histone deacetylase (HDAC, a promising target for cancer treatment) inhibition and antiproliferative activity toward cervical tumor [2]. The 5-methoxy isatin thiosemicarbazone with the N(4) substitution by hydroxamic acid has been reported as prominent carbonic anhydrase inhibitor [3]. In the thiosemicarbazone of isatin and other carbonyl compounds, the presence of sulphur atom, aromatic heterocyclic moiety and N(4) modification by stable groups such as allyl, benzyl or heterocyclic ring (morpholinyl, pyrollidinyl, *etc*.) have been found to increase the anticancer activity toward various cancer cell lines [4]. Beside isatin thiosemicarbazone, other α-*N*-heterocyclic thiosemicarbazones (HCTs) are the most potent inhibitor of ribonucleotide reductase (RR) enzyme that have been studied as antitumor agents toward leukemia, breast, non-small cell lung, pancreatic, bladder, prostate and cervical cancer cell lines [5]. 4-(Dimethylamino)-benzaldehyde thiosemicarbazone exhibited significantly higher inhibitory activity (IC_{50}, < 1) than that of standard drug catechin (IC_{50}, 2.8 μM) toward the aggregation of amyloid-β, with the decrease in formation of fibrils [6]. 2-Pyridine formamide thiosemicarbzones with N(4) modifications induced

apoptosis and showed significant cytotoxicity against human pancreatic cancer cell (PANC-1) in nutrient deprived medium (NDM) [7]. Copper(II) complex of isatin-Schiff base *viz.* (*E*)-1-methyl-3-(phenylimino)indolin-2-one and ligand itself have found to induce cell death in p53-positive tumors and activate the pro-apoptotic PUMA gene for apoptosis stimulation in breast cancer [8]. Copper(II) complexes have been found as more active antitumor agents than those of Fe(III), Pt(II), *etc.* because of the oxidation-reduction behaviour of copper and 4-coodinated planar geometry that facilate them to attach with the nitrogen base of DNA, thus blocking the base replication leading to the inhibition of tumor growth as compared to that of six-coordinate Fe(III) complexes [4,9]. The *in vitro* antiproliferative study and apoptosis mechanism of copper(II) complexes of N(4) substituted 2-acetylpyridine-3-thiosemicarbazone against A549 and Caco-2 cell lines showed that these complexes have higher antitumor activities (IC_{50} 0.20-0.42 μM, A549) and (IC_{50} 0.068-1.07 μM, Caco-2) than that of their corresponding ligands (IC_{50}, 1.07-11.77 μM, A549) and (IC_{50}, 0.75-86.89 μM, Caco-2) with the promotion of cell apoptosis by increasing the reactive oxygen species (ROS) generation. In addition, the N(4) modification of thiosemicarbazone by methyl group has been reported to enhance the anticancer activity of ligands [10]. The *in vitro* cytotoxic study of copper(II) complex of 5-nitroisatin thiosemicarbazones against HCT 116 exhibited higher dose dependent anticancer potency than that of standard, 5-fluorouracil with DNA cleavage *via* oxidative and hydrolytic pathways [11].

The growing research works on thiosemicarbazones and their Cu(II) complexes made us avid for designing such type of N(4) substituted thiosemicarbazone and its Cu(II) complex and study their anticancer activity toward various cancer cell lines.

EXPERIMENTAL

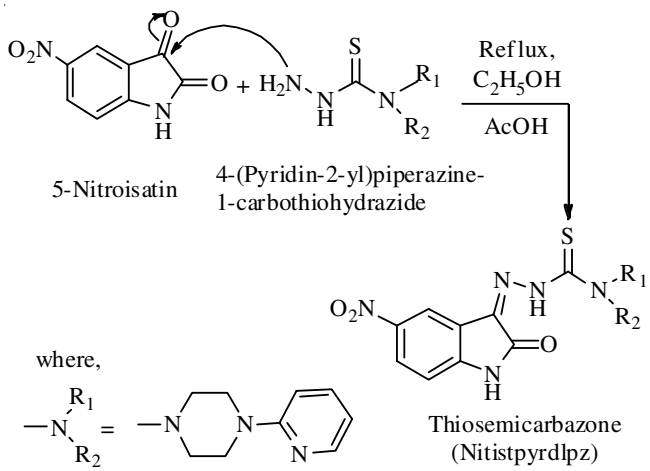
N-Methyl aniline and 5-nitroisatin (Alfa Aeser), sodium chloroacetate (Chemical Center, India), carbon disulphide (Qualigens Fine Chemicals), hydrazine hydrate, 98% (Fisher Scientific), methyl alcohol (Fisher Scientific), 1-(2-pyridyl)-piperazine (Spectrochem Pvt. Ltd. India), diethyl ether (Merck), acetonitrile (Merck), absolute alcohol (Changshu Hongsheng fine chemical), concentrated hydrochloric acid (Merck), glacial acetic acid (Fisher Scientific), copper(II) chloride (Merck) and sodium hydroxide (Fisher Scientific) were of reagent grade and used without further purification.

Melting points of the compounds were confirmed on melting point apparatus (Philip Harris). CHN analysis of the ligand 5-nitroisatin-4-(1-(2-pyridyl)piperazinyl)-3-thiosemicarbazone (Nitistpyrdlpz) on Perkin-Elmer CHN-2400 was carried out at IIT Madras, Chennai. CHN analysis of copper(II) complex (Cu-Nitistpyrdlpz) on ThermoFinnigan elemental analyzer, UV-visible spectroscopic measurements on UV-1800, SHIMADZU UV-spectrophotometer using DMSO solution (25 μM) in the range of 700-250 nm, FTIR on Tensor-37 FTIR spectrophotometer from the wave number 4000 to 400 cm^{-1}, optical bench detector and platinum attenuated total reflectance (Pt-ATR), nuclear magnetic resonance (NMR) spectroscopic measurements in DMSO-d_6 at room temperature on Bruker advance III HD NMR 400 MHz spectrometer, high resolution electron spray ionization mass spectrometry (ESI-HRMS) on Bruker IMPACT HD liquid chromatography mass spectrometer and thermogravimetric analysis (TGA) on simultaneous thermal analyzer-SDT 650, DSC/TGA system under a dynamic nitrogen atmosphere in the 20-800 ºC temperature range at a heating rate of 10 ºC min^{-1} were carried out at the Department of Chemistry, Savitribai Phule Pune University, Pune, India.

Powder X-ray diffraction patterns (PXRD) of the copper complex was recorded on High resolution D8 Discover Bruker diffractometer having point detector (scintillation counter) and monochromatized CuKα1 radiation with scan rate of 1.0 second/step and step size 0.02º at 298 K in the range of 2θ = 5-80º at Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur, Nepal. The Le-Bail fitting of the observed PXRD pattern for the copper complex was determined by comparing the crystal parameters of similar copper(II) thiosemicarbazones with CCDC no. 1864662. Electron paramagnetic resonance (EPR) spectral data were recorded on Bruker biospin corp. (EMX series) Model: A 200-9.5/12B/S at Sogang University, Seoul, Korea.

Synthesis of N(4) substituted thiosemicarbazone and its copper(II) complex

Synthesis of thiosemicarbazone: 1-(2-Pyridyl)piperazinyl substituted thiosemicarbazide *viz.* 4-(pyridin-2-yl)piperazine-1-carbothiohydrazide was synthesized by the method of Scovill [12]. Equimolar mixture of 5-nitroisatin and 4-(pyridin-2-yl)-piperazine-1-carbothiohydrazide (2 mmol each) in 20 mL of absolute ethanol was refluxed at 80 ºC in presence of catalytic amount of glacial acetic acid for 6 h to obtain thiosemicarbazone (Nitistpyrdlpz) (**Scheme-I**) [13]. The resulting mixture was cooled at room temperature, filtered, washed with cold absolute ethanol and then dried in oven at 50 ºC overnight. Yield: 90%; colour: yellow; m.p.: 252 ºC; anal. calcd. (found) % for: $C_{18}H_{17}N_7O_3S$ (*m.w.* 411.44): C, 52.55 (52.32); H, 4.16 (3.90); N, 23.83 (23.41). ESI-HRMS: $[M+H]^+$ displays a peak at *m/z* 412.1187 (calcd. *m/z* 412.1186]



Scheme-I: Synthesis of 5-nitroisatin-4-(1-(2-pyridyl)piperazinyl)-3-thiosemicarbazone

and $[M+Na]^+$ displays a peak at *m/z* 434.1004 (calcd. *m/z* 434.1005]; UV-Visible (solvent: DMSO (25 µM); λ_{max}: nm): 324, 359, 461.

Synthesis of copper(II) complex of thiosemicarbazone (Cu-Nitistpyrdlpz): Equimolar mixture of 5-nitroisatin-4-(1-(2-pyridyl)piperazinyl)-3-thiosemicarbazone (Nitistpyrdlpz) and $CuCl_2 \cdot 2H_2O$ (2 mmol each) in 15 mL absolute ethanol was refluxed at 80 ºC for 6 h to obtain greenish brown precipitate of copper(II) thiosemicarbazone *viz.* (((*Z*)-((*E*)-(5-nitro-2-oxoindolin-3-ylidene)hydrazono)(4-(pyridin-2-yl)piperazin-1-yl)methyl)thio)copper(II) chloride (Cu-Nitistpyrdlpz). The compound thus formed (**Scheme-II**) was cooled at room temperature, filtered, washed with cold absolute alcohol followed by diethyl ether, recrystallized in absolute alcohol, dried in oven at 50 ºC overnight and finally at 80 ºC for 2 h [14]. Yield: 90%; colour: greenish brown; m.p.: 264-266 ºC; anal. calcd. (found) % for: $C_{18}H_{16}N_7O_3SClCu$ (*m.w.* 509.43): C, 42.44 (42.38); H, 3.17 (3.60); N, 19.25 (19.16). ESI-HRMS: $[M+H]^+$ displays a peak at *m/z* 509.0091 (calcd. *m/z* 509.0092] and $[M+Na]^+$ displays a peak at *m/z* 530.9909 (calcd. *m/z* 530.9912]; UV-Visible (solvent: DMSO (25 µM); λ_{max}: nm): 357, 430; EPR: $g_{\parallel} = 2.899$, $g_{\perp} = 2.101$, g_{av} or $g = 2.367$.

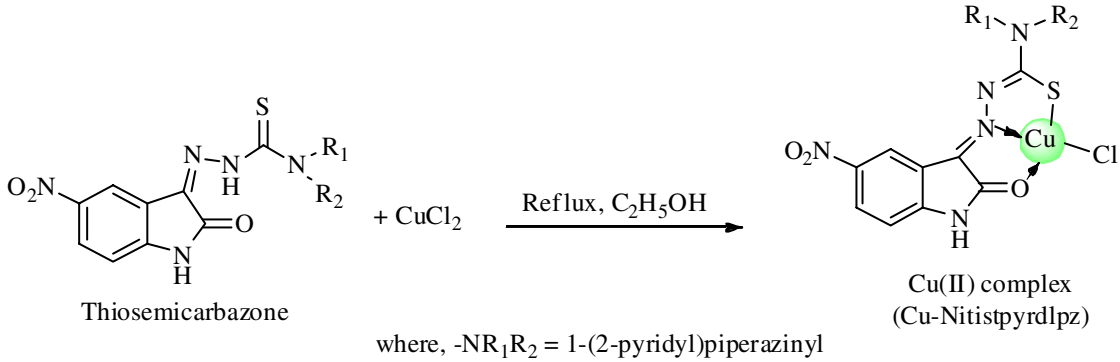
Cell viability assay: MDA-MB-231, MCF-7 and A431 cell lines were purchased from National Center for Cell Science, Pune, India. Cells were cultured in DMEM media, purchased from Invitrogen (California, USA) supplemented with 10,000 units/mL, penicillin and 10 mg/mL streptomycin in 0.9% normal saline, purchased from HIMEDIA and 10% heat-inactivated fetal calf serum purchased from Invitrogen (California, USA). Cell culture grade DMSO purchased from HIMEDIA was used to dissolve the drug and dilution preparation.

The synthesized thiosemicarbazone and its copper (II) complex were screened for anticancer activity against breast cancer cell lines MDA-MB-231 and MCF-7 and epidermoid carcinoma A431 using cell viability assay. Cells were maintained in DMEM medium supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 ºC in 5% CO_2 incubator.

A 3000 cells/100 µL were seeded in each well and treated with defined concentration of synthesized thiosemicarbazone and its copper(II) complex dissolved in DMSO. For determining viability percentage, cells were washed with PBS following media removal. Cells were incubated with 50 µL of 0.5% crystal violet on rocker (20 oscillations per min) for 30 min and then washed three times with PBS. The excess crystal violet was removed by pipetting out and plates were left undisturbed for overnight drying. The dried wells were incubated with 200 µL of methanol for 30 min at room temperature in shaking condition. The optical density was measured at 570 nm using microplate reader. The percentage inhibition of the cells was calculated on the basis of percentage viability. The results were expressed as mean value of four independent measurement performed under identical conditions with standard deviation.

RESULTS AND DISCUSSION

IR analysis: The assignments of FTIR diagnostic bands of thiosemicarbazone and its copper(II) complex are listed in Table-1. In the synthesized thiosemicarbazone (Nitistpyrdlpz), a broad absorption band located at 3132 cm^{-1} attributed to the stretching vibration of indole; ν(N-H) and thioamide; ν(N-H) [15]. The degree of hydrogen bonding, molecular symmetry, molecular complexity and substitution on nitrogen atoms of thiosemicarbazone affect the stretching vibration of ν(N-H) [16]. In thiosemicarbazone, the existence of stable six-membered hydrogen bonded rings as a result of intramolecular thioamide-N–H···O=C (indole) bonds caused to show the spectra of ν(N-H) at 3140 cm^{-1} which is comparable to that of synthesized compound (3132 cm^{-1}) [16,17]. The $–NO_2$ group present in the synthesized thiosemicarbazone experienced higher inductive (–I) and resonance (–R) effect than that of –F, –Cl, –Br, $–OCH_3$ of repective molecules. As a result the former molecule caused to shift the ν(N-H) frequency in positive mode instead of negative mode as observed in later compounds [18]. The nature of substituent present at N(4) position of thiosemicarbazone also affects the frequency of ν(N-H) [16]. Thus the effects of substitution, –I, –R and hydrogen bonding may result the appearance of broad single band for both indole; ν(N-H) and thioamide; ν(N-H). In the complex, thioamide N-H deprotonate to form thiosemicarbazone anion and the band in uncomplexed thiosemicarbazone attributed to indole ν(N-H) vibration mode was shifted to lower frequency (3098 cm^{-1}) [19,20]. The high –I and –R effects by $–NO_2$ weakened the hydrogen bonding of thiosemicarbazone and caused to decrease the chance of vibrational coupling and elevated the wave number specific to indole ν(N-H). Thus an additional single broad band assigned to the stretching vibration of indole ν(N-H) was seen at 3359 cm^{-1} in the complex (Cu-Nitistpyrdlpz) [18].



Scheme-II: Synthesis of copper(II) complex of 5-nitroisatin-4-(1-(2-pyridyl)piperazinyl)-3-thiosemicarbazone

TABLE-1
FTIR SPECTROSCOPIC DATA (cm^{-1}) OF Nistpyrdlpz AND Cu-Nitistpyrdlpz

Compounds	ν(N-H)	ν(C=O)	ν(C=N)	ν(NO_2)	ν(N-N)	ν(C=S)
Nistpyrdlpz	3132 m (Indole + azomethine)	1710 s	1595 s	1521 s	1161 s	1385 m 836 s
Cu-Nitistpyrdlpz	3359 w 3098 w (Indole)	1602 s	1527 s	1492 s	1176 s	1242 m 815 s

In the ligand, two foremost bands of stretching vibrations, due to ν(C=O) group of cyclic amide in 5-nitroisatin moiety and azomethine, ν(C=N) of thiosemicarbazone were observed at 1710 and 1595 cm^{-1} respectively [20]. In the complex, Cu(II) is coordinated by the O–atom of C=O and N-atom of C=N with the lowering of frequency, so the two sharp bands due to the stretching vibrations of ν(C=O) and ν(C=N) were identified at 1602 and 1527 cm^{-1}, respectively [21]. The $–NO_2$ group of the thiosemicarbazone and its Cu(II) complex is planar due to having sp^2 hybridized nitrogen atom and π-bonds. It makes the normal mode frequency of Ar-NO_2 sensitive for conjugation with aromatic ring. As a result, the $–NO_2$ group bonded to conjugated system absorbs energy at lower frequencies than that in non-conjugated system, so the strong absorption band was seen at 1521 cm^{-1} in free Nitistpyrdlpz and 1492 cm^{-1} in Cu-Nitistpyrdlpz attributed to the asymmetric stretching of ν(NO_2) [22]. The absence of band at 2575 cm^{-1} corresponding to the stretching vibration of ν(S-H) [23] and presence of two strong bands at 1385 and 836 cm^{-1} corresponding to the stretching vibration of ν(C=S) indicates that thiosemicarbazone exists in thio-keto form [11,20,24]. The two IR bands attributed to ν(C=S) of ligand shifted to lower frequency (1242 cm^{-1}, 815 cm^{-1}) on coordinated to copper(II) ion [24]. A sharp absorption band present at 1161 cm^{-1}, assigned to the stretching vibration of ν(N-N) in uncomplexed ligand [24,25] shifted to slightly higher frequency (1176 cm^{-1}) in its copper(II) complex [25]. In the complex, the positive mode of ν(N-N) band may arise because of the increasing bond strength *i.e.* increasing π-electrons density between these atoms as a result of deprotonation at H-N-C=S during the coordination of thiosemicarbazone anion to copper(II) ion [24]. The coordination from three coordinating groups *viz.* C=N (azomethine), C=O (isatin moiety) and C-S (thiosemicarbazone anion) to copper(II) ion along with the one coordination site being occupied by Cl^- indicates that copper(II) complex exhibits the distorted square planar geometry.

NMR analysis: The chemical shift (δ, ppm) values of N(4) substituted thosemicarbazones are summarized in Table-2. In the ^{1}H NMR (400 MHz, DMSO-d_6) spectrum, the absence of sharp signal at 4 ppm assigned to thiol (-SH) proton indicates that the Schiff's base ligand (Fig. 1) exists as neutral thio-ketone form in solution [26].

Two sharp singlets present at 13.04 ppm and 11.90 ppm are ascribed to the highly polar thiosemicarbazide N-NH *viz.* (N3-H) and indole N-H *viz.* (N1-H), respectively [11,27]. The peaks for aromatic protons were seen in the region 8.23-6.67 ppm. Two aromatic protons (C4-H, C6-H) of indole moiety were seen as high intensity multiplet peak at 8.23 ppm that can be

TABLE-2
^{1}H NMR AND ^{13}C NMR SPECTRAL DATA (ppm)
OF Nitistpyrdlpz (400 MHz, DMSO-d_6)

Proton	δ (ppm)	Carbon	δ (ppm)
Thiosemicarbazide N-NH *viz.* -N(3)H	13.04, s	C=S *viz.* -C(10)	179.17
Indole N-NH *viz.* -N(1)H	11.90, s	C=O *viz.* -C(2)	163.94
-H(4), -H(6)	8.23, m	-C(15)	158.52
-H(19)	8.13, d	-C(19)	147.94
-H(7)	7.57, d	-C(5)	147.33
-H(17)	7.15, dd	-C(9)	143.26
-H(16)	6.84, d	-C(17)	138.17
-H(18)	6.67, dd	C=N *viz.* -C(3)	132.50
-H(12), -H(13)	4.15, t	-C(6)	127.07
-H(11), -H(14)	3.75, t	-C(4)	121.33
–	–	-C(8)	115.68
–	–	-C(7)	113.62
–	–	-C(18)	111.84
–	–	-C(16)	107.10
–	–	-C(12), -C(13)	49.84
–	–	-C(11), -C(14)	43.76

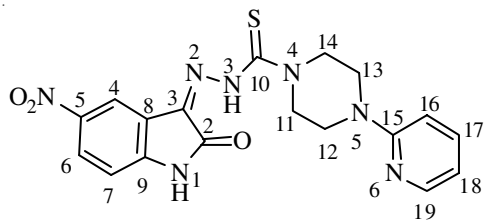


Fig. 1. Structure of thioketone form of Nitistpyrdlpz

seen as two separate peaks at high resolution and other aromatic protons of indole and 1-(2-pridyl)piperazine moieties were seen as doublet peak at 8.13 ppm (C19-H), doublet peak at 7.57 ppm (C7-H), doublet of doublets peak at 7.15 ppm (C17-H), doublet peak at 6.84 ppm (C16-H) and doublet of doublets peak at 6.67 ppm (C18-H) [11,27,28]. The two triplet signals observed at 4.15 ppm (C12-H, C13-H) and 3.75 ppm (C11-H, C14-H) are attributed to aliphatic protons of 1-(2-pridyl)piperazine moiety [20,28]. The ^{13}C NMR spectrum exhibited three distinct downfield peaks at 179.17, 163.94 and 132.50 ppm corresponding to carbon atoms of C=S, C=O and C=N, respectively [11,27,29]. The peaks for aromatic carbon atoms of isatin moiety were observed at 147.33 ppm (C5), 143.26 (C9), 127.07 ppm (C6), 121.33 ppm (C4), 115.68 ppm (C8), 113.62 ppm (C7) [27,29]. The two distinct peaks of more polar aromatic carbon atoms (C15 and C19) of 1-(2-pyridyl)piperazine moiety were observed at 158.52 ppm and 147.94 ppm, respectively while other aromatic carbon atoms were observed at 138.17 ppm (C17), 111.84 ppm (C18) and 107.10 ppm (C16) [28]. The peaks for

two non-equivalent carbon atoms of piperazinyl ring were seen at 49.84 ppm (C12, C13) and 43.76 ppm (C11, C14) [20,28].

Mass analysis: The high resolution electron spray ionization mass spectrometry (ESI-HRMS) study for the measurement of mass spectrum of thiosemicarbazone (Nitistpyrdlpz) and its Cu(II) complex (Cu-Nitistpyrdlpz) was carried out in positive mode (mode at which the analyte is sprayed at low pH to encourage positive ion formation). The ligand showed the mass spectral peak of protonated molecular ion $[M+H]^+$ at *m/z* 412.1187 amu (calcd. 412.1186) [30] and that of $[M+Na]^+$ at *m/z* 434.1004 amu (calcd. 434.1005) [31,32]. The complex exhibited the mass spectral peak of protonated molecular ion $[M+H]^+$ at *m/z* = 509.0091 amu (calcd. 509.0092) and that of $[M+Na]^+$ at *m/z* = 530.9909 amu (calcd. 530.9912) [32]. In the complex, an additional peak, adjacent to molecular ion $[M+Na]^+$ peak has been observed because the copper atom has two isotopes *viz.* ^{63}Cu and ^{65}Cu. The mass spectral data confirms the proposed molecular formulae of ligand and its copper(II) complex.

UV-visible analysis: The electronic absorption spectra of synthesized compounds were measured at 700-200 nm using 25 µM of DMSO solution. In thiosemicarbazone, a broad absorption band was observed at 324 nm due to $\pi\rightarrow\pi^*$ electronic transition of –C=C- (may be of benzene or pyridyl ring) and two broad bands with shoulder were observed at 359 nm and 461 nm due to $n\rightarrow\pi^*$ electronic transition of-C=N, -C=O and -HN-C=S (thioamide) group [33,34]. On complexation with Cu^{2+} ion, the intraligand absorption band due to $n\rightarrow\pi^*$ electronic transition (359 nm) showed marginal change and was seen at 357 nm [35,36]. The band (461 nm) of uncomplexed thiosemicarbazone was shifted to lower wavelength in its complex and merged with the broad band of higher energy $n\rightarrow\pi^*$ electronic transitions (357 nm) [37,38]. In the complex, the coordination of terdentate thiosemicarbazone anion to Cu^{2+} ion occurs through the O-atom, N-atom and S^- of C=O, C=N and C-S^- groups, respectively. The coordination from S^- to Cu^{2+} ion resulted the ligand to metal charge transfer (LMCT). As a result a high intensity band was observed at the wavelength 430 nm in the complex [39]. The thiosemicarbazone is a weak field ligand, so the crystal field splitting of copper is very small with high spin and as a result the absorption band specific to *d-d* transition was not seen in the complex. It may be overlapped with the appeared broad band or hidden because of the solvent effect of DMSO [32].

Thermal studies: Thermogravimetric analysis (TGA) of copper(II) complex of thiosemicarbazone (Cu-Nitistpyrdlpz) was performed in the atmosphere of nitrogen in the temperature range of 20-800 ºC at a linear heating rate of 10 ºC/min and the data are shown in Table-3. The complex exhibits the endothermic pattern of decomposition [40]. The TGA pattern due to the loss of coordinated or hydrated water molecule was not seen, so the copper(II) complex is anhydrous and stable in air at room temperature, which is in accordance with the elemental as well as spectral analysis. The TGA pattern showed the gradual fragmentation and thermal decomposition of organic ligand above 250 ºC [41]. At 255.70 ºC, the decomposition of thiosemicarbazone and isatin moiety was observed in the form of (C_3HN_3O) [found/calcd. 18.87/18.88]. The decomposition of 1-(2-pyridyl)piperazine moiety ($C_2H_4N_2$) and the part of complex (ClS) were observed at 313.46 ºC [found/calcd. 11.55/11.00] and 422.56 ºC [found/calcd. 12.99/13.25], respectively. Above 700 ºC, the TGA graph looked like a plateau due to the formation of stable CuO as residual substance [42].

PXRD studies: The Le-Bail fitting, a technique useful for extracting intensities and refining the unit cell of the complex [43,44] was performed by using the crystal parameters of similar compound, CCDC no. 1864662 [20]. On the basis of Le-Bail fitting pattern (Fig. 2), the complex has monoclinic $P12_11$ symmetry in which the ligand (Nistpyrdlpz) is coordinated in a tridentate manner through oxygen (O), nitrogen (N) and sulphur (S) and the fourth coordination site is occupied by chloride ion. The lattice constants obtained after the Le-Bail fitting are shown in Table-4. The PXRD pattern of the complex suggested the affirming distortion from perfect square planar geometry [45,46] with one side occupied by the counter Cl^- anion.

EPR studies: The EPR study of polycrystalline copper(II) thiosemicarbazone (Cu-Nitistpyrdlpz) was accomplished on X-band at 9.8610 GHz under room temperature to give the information about the oxidation state of copper atom in the complex, ligand to copper coordination environment, geometries of the complexes through the study of its crystal field splitting, ground state and g value calculation [47]. The EPR spectrum

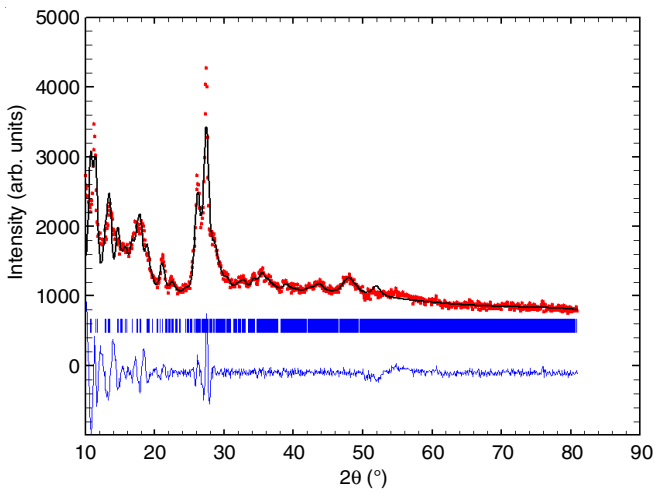


Fig. 2. Le-Bail fitting of the (Cu-Nistpyrdlpz) complex

TABLE-3
THERMAL DECOMPOSITION DATA OF Cu-Nitistpyrdlpz

Stage	Peak temperature in TGA (°C)	TGA mass loss (%)	Theoretical mass loss (%)	Fraction loss/residue
First	255.70	18.87	18.88	C_3HN_3O
Second	313.46	11.55	11.00	$C_2H_4N_2$
Third	422.56	12.99	13.25	ClS
Fourth	700.10	–	–	CuO as residue

TABLE-4
LE-BAIL FITTING PARAMETER OF Cu-Nistpyrdlpz COMPLEX

Parameter	Cu(Nitispyrdlpz)Cl
Crystal system	Monoclinic
Space group	$P12_11$
a (Å)	8.3166
b (Å)	22.5896
c (Å)	10.2941
α (°)	90.0000
β (°)	97.8373
γ (°)	90.0000

(Fig. 3) of the complex exhibited axial symmetry with broad signal because the values of $g_{\parallel}$ and $g_{\perp}$ are higher than that for a free electron ($g_{\parallel} > g_{\perp} > 2.0023$) [13]. This trend of g values; $g_{\parallel}$ (2.899), $g_{\perp}$ (2.101) and g_{av} or g (2.367) showed that the complex is mononuclear and contains Cu(II) ion with one unpaired electron which is localized in $d_{x^2-y^2}$ orbital [48,49] and is in agreement with the existence of square planar or square pyramidal Cu^{2+} systems with axially symmetrical d^9 copper(II) complex having tetragonal elongation along the *z*-axis [50,51]. As supported by other spectroscopic measurements, the copper(II) complex considered as distorted square planar geometry.

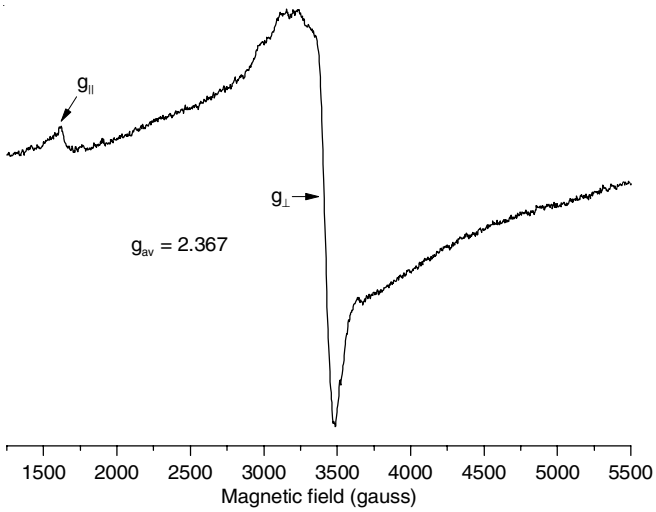


Fig. 3. EPR spectrum of Cu-Nitistpyrdlpz complex

Anticancer activity: The cell viability results (Table-5) showed that copper(II) complex (Cu-Nitistpyrdlpz) has significant potential to reduce the percentage of cell viability in MCF-7, MDA-MB-231 and A431 cell lines while that of ligand (Nitistpyrdlpz) has almost same as that of control. Thus the complex is found to be better anticancer agent than its ligand.

Copper complexes have ability to induce apoptosis in cancer cells by enhancing the production of ROS because they have biologically accessible redox potentials, high affinities toward nucleobase and high intercalating efficiencies toward protein and DNA [52,53]. The copper(II) complex (Cu-Nitistpyrdlpz), not only can show significant anticancer activity, but can also induce the apoptosis of cancer cell directly *via* multiple mechanisms, such as telomerase activity inhibition and activating apoptic pathway through protein regulation (cyclin and cyclin dependent kinases down regulation and cyclin dependent

TABLE-5
CELL VIABILITY (μM) AGAINST
CANCER CELL LINES FOR 72 h

Compounds	Cell viability (normalized with non-treated sample taken as 1)		
	MCF-7	MDA-MB-231	A431
Nitistpyrdlpz	0.95 ± 0.14	0.97 ± 0.08	0.98 ± 0.06
Cu-Nitistpyrdlpz	0.47 ± 0.14	0.36 ± 0.05	0.49 ± 0.02

kinase up regulation) [54]. The copper(II) complexes with planar structure have been found to show high efficiency to DNA damage or nuclease activity [55] mainly through reactive oxygen species (ROS) production where ligands of the complexes are believed to neutralize the charge of metal ions, enhance lipophilicity of the complexes to facilitate their transport *via* cell membrane and bind to DNA or interact with proteins [56]. Copper complexes have been found to show significantly different mechanism of action than that of standard drug cisplatin *e.g.* casiopeins exhibited high antineoplastic activity by inhibiting respiration and ATP synthesis [57]. The copper(II) complexes of N-substituted isatin thiosemicarbazones exhibited promising cytotoxic effect against Jurkat cells, HeLaS3 through the inhibition of cancer cell growth through apoptosis [58,59].

The *in vitro* inhibition study shows effect (Tables 5 and 6) of synthesized thiosemicarbazone (Nitistpyrdlpz) and its Cu(II) complex (Cu-Nitistpyrdlpz) toward breast cancer cell lines MCF-7 and MDA-MB-231 and epidermoid carcinoma A431. The metal complex (Cu-Nitistpyrdlpz) exhibited higher anticancer activity by greatly inhibiting the cell growth in MCF-7, MDA-MB-231 and A431 at micromolar concentration (IC_{50} 0.85-1.24 μM). Viability of 47% and 14% was observed in MCF-7 and MDA-MB-231 post 72 h of treatment in reference to control cells. A431 upon treatment exhibited 49% cell viability in reference to control cell. In contrast the metal free complex didn't show any inhibition in growth as all cells grew above 95% in respect to normal. Thus, it is apparent that conjugation of the thisemicarbazone with the metal significantly enhances its potential to inhibit proliferation. All the cell lines showed a significant reduction in their proliferation when treated with Cu-Nitistpyrdlpz. Growth inhibition was most significant in the breast cancer cell line MD-AMB-231 by Cu-Nitistpyrdlpz.

TABLE-6
IC_{50} (μM) VALUES OBTAINED WITH
DIFFERENT CELL LINES FOR 72 h

Compounds	Antitumor activity, IC_{50} (μM)		
	MCF-7	MDA-MB-231	A431
Nitistpyrdlpz	3.28 ± 0.14	3.52 ± 0.05	3.53 ± 0.02
Cu-Nitistpyrdlpz	1.24 ± 0.14	0.85 ± 0.08	1.00 ± 0.06
Control	2.60-3.42	2.32-3.62	2.04-3.52

Conclusion

Mononuclear copper(II) complex of N(4) modified thiosemicarbazone *i.e.* Cu-Nitistpyrdlpz has been synthesized and the distorted square planar geometry of the complex was confirmed. The copper(II) complex reduced the percentage of cell viability remarkably higher than that of thiosemicarbazones

toward MDA-MB-231, A431 and MCF-7 cell lines. The complex exhibited anticancer activity at micromolar concentration toward both breast and skin cancer cell lines (IC_{50} 0.85-1.24 μM). The Cu-Nitistpyrdlpz complex may be used as non-platinum anticancer drug against MDA-MB-231 (IC_{50} 0.85 μM).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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