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Metal (II) Complexes as Potent Apoptosis Inducers in Testicular Germ Cells of *Capra hircus*

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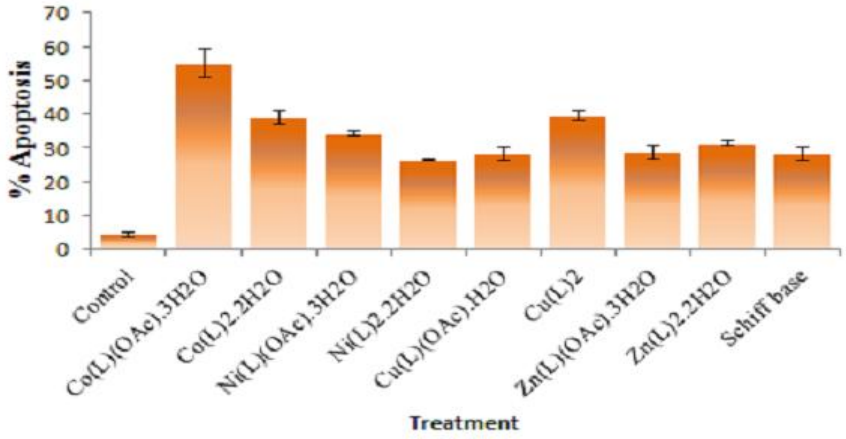
ABSTRACT

Metal (II) complexes (Co, Ni, Cu, Zn) of an imine were synthesized. The synthesized compounds were characterized by analytical, spectroscopic and thermal techniques and evaluated for their apoptosis-induction potential in testicular germ cells of goat. In situ end labeling (TUNEL) assay demonstrate that the complexes exhibit increased incidence of DNA fragmentation. The current findings suggested that apoptosis comprised major mode of cell death by metal complexes.

Graphical Abstract:



Keywords: Apoptosis, fluorescence, metal complexes, pyrazole, Schiff base.

INTRODUCTION

The design of novel compounds which can selectively bind DNA and induce apoptosis is of high importance for research in chemical biology and medicinal chemistry offering unique targets for chemotherapeutic intervention [1-4]. The use of transition metal complexes in chemotherapy increasingly

rely on apoptosis [5]. The metal ions are well known to enhance the efficiency of a therapeutic agent upon coordination and accelerate the drug action [6, 7]. A perusal of literature reveals that many new strategies targeting apoptosis with non-platinum metal complexes [8, 9] are feasible and may be used in the treatment of cancer. A number of metal complexes exhibiting cytotoxic activity through cell apoptosis have been studied [10-12]. More recently Zn(II) complexes were found to be inducers of apoptosis by triggering DNA-damage mediated p^{53} phosphorylation in cancer cells [13].Some Schiff base Cu(II) complexes have been reported as inducers of apoptosis in human cancer cells [14]. Current interest in complexes derived from heterocyclic nitrogen derivatives is owing to their antibacterial, antifungal, anti-inflammatory and antitumor activities [15-24].

Appreciating these findings, we synthesized new transition metal complexes from pyrazole and triazine derivatives and interestingly, we find that all the newly synthesized compounds are inducers of apoptosis and may thus influence therapeutic strategy.

MATERIALS AND METHODS

Analytical grade reagents were used for the present work. 4-amino-3-mercapto-6-methyl-5-oxo-1,2,4-triazine [25] and 3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazole-4-carboxaldehyde[26] were prepared by reported literature methods. The metal estimation was carried out using standard gravimetric methods (cobalt was determined as cobalt pyridine thiocyanate, nickel as nickel dimethyl glyoximate, copper as cuprous thiocyanate and zinc as zinc ammonium phosphate [27]. The analytical data agreed well with the proposed structures of the complexes. Thermo Scientific (FLASH 2000) CHN Elemental Analyzer at SAIF, Panjab University, Chandigarh was used for CHN analysis. The IR spectrometer (MB-3000 ABB Spectrometer) in the range 4000-250 cm^{-1} was employed for recording IR spectra. A Bruker ACF 300 NMR spectrometer at 300 MH_Z was used to record ^1H NMR spectra using DMSO-d_6 as the solvent. Thermal analyses of metal complexes were carried out on a Perkin Elmer (Pyris Diamond) instrument in atmospheric air at 10°C Min^{-1}. SHIMADZU RF-5301 PC spectrophotometer was used to record the fluorescence spectra. The X-Band ESR spectra were recorded at a frequency of 9.1 GHz on a Varian E-112 ESR spectrometer under the magnetic field 3000 Gauss at SAIF, IIT Bombay.

Synthesis of Schiff base: 4-[(3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methyleneamino]-3-mercapto-6-methyl-5-oxo-1,2,4-triazine (Figure 1)0.5 g (1.155 mmol) of 4-amino-3-mercapto-6-methyl-5-oxo-1,2,4-triazine in dried methanol was added to 0.33 g (1.155 mmol) of 3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazol-4-carboxaldehyde in methanol. The reaction mixture was refluxed for 9h and then the volume was reduced on rotavapor. The solid formed was filtered, washed with ice cold methanol and recrystallized. It is Light yellow. Yield 77%; m.p: 244-246°C.

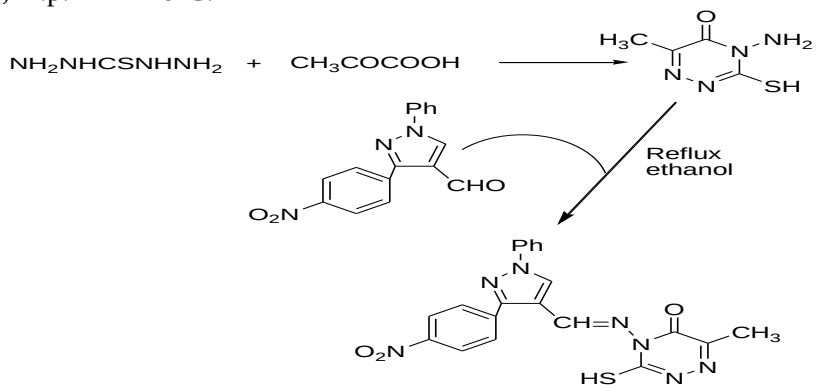


Figure 1 Scheme for synthesis of Schiff base

Synthesis of 1:1 Metal complexes: The hot ethanolic solutions of 0.12 g (0.46 mmol) Co(II) acetate, 0.12 g (0.46 mmol) Ni(II) acetate, 0.09 g (0.46 mmol) Cu(II) acetate and 0.11 g (0.46 mmol) Zn(II) acetate were treated with the hot ethanolic solutions of 0.2 g (0.46 mmol) of the ligand. The solid precipitated immediately was filtered and washed successively with warm water, ethanol and acetone.

Synthesis of 1:2 Metal complexes: The 1:2 metal complexes were synthesized by adding ethanolic solution of Co(II) acetate (0.12 g, 0.46 mmol), Ni(II) acetate (0.12 g, 0.46 mmol), Cu(II) acetate (0.09 g, 0.46 mmol) and Zn(II) acetate (0.11 g, 0.46 mmol) respectively to 0.4 g (0.92 mmol) ethanolic solutions of the ligand separately with stirring. The solid complexes precipitated immediately were filtered, washed with warm water followed by ethanol and acetone.

Apoptotic assays (*in vitro)*

Reagents/chemicals: DMSO, DMEM, phosphate buffer saline (PBS), antibiotics (penicillin, streptomycin), acridine orange, haematoxylin, eosin and *in situ* apoptosis detection kit.

Collection of materials and *in vitro* culture: Goat (*Capra hircus*) testes were procured from the slaughter houses around Kurukshetra (26º60 N, 76 º50 E), India and brought to laboratory in normal saline at 4ºC. Then the testes were de-capsulated and cut into smaller pieces. The testicular tissue pieces were washed with phosphate buffer saline and were incubated with the compounds to be tested (at 10µM concentration) in Dulbecco's modified Eagle's Medium (DMEM) supplemented with antibiotics (200 units having penicillin and streptomycin each at 100 $IUmL^{-1}$ concentration) for 6 h in CO_2 incubator (5% CO_2, 95% humidity, 38ºC) for *in vitro* culture.

Histo-morphological analysis: The treated testicular tissue was fixed in Bouin's fixative for 24 h and then washed with tap water. It was then dehydrated using ethanol and xylene followed by its embedding in paraffin wax. Blocks were prepared from embedded tissues and serially trimmed at 5µm using microtome. The sections were stretched on albumin coated slides and poly-l-lysine coated slides for TUNEL assay followed by its air drying. For histomorphological analysis, tissue sections on albumin coated slides were rehydrated and dehydrated using graded series of ethanol and stained with haematoxylin and eosin.

Apoptosis detection assays

Fluorescence assay: The treated testicular tissue, of mature goat from *in vitro* culture, was used to harvest testicular germ cells using phosphate buffer saline (PBS). The cell suspension was plated at a density of 1 X 10^5 $cellsmL^{-1}$ and harvested cells were washed twice using PBS and GeNeI™ centrifuge. Then, apoptosis was assayed in cell suspension of testicular tissue by acridine orange staining under the fluorescence microscope (Olympus, Japan) with 500-525 nm filters. Cells showing red fluorescence were characterized as normal with intact membrane whereas the cells exhibiting green fluorescence were considered as apoptotic.

***In situ* end labeling (TUNEL) assay:** Treated stretched sections on Poly-L-Lysine coated slides were used for TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay using TACS® 2 TDT-DAB Apoptosis Detection Kit. The sections were hydrated using graded series of ethanol, fixed and immobilized in PBS. The slides were permeabilized using Proteinase K solution at room temperature to allow the enzyme and stains enter into the cells. This was followed by quenching using hydrogen peroxidase to block endogenous peroxidase activity. The sections were labeled with TDT (Terminal deoxynucleotidyl transferase) reaction mixture and further incubated with streptavidin-HRP conjugate for amplification. After washing twice in PBS, sections were stained with DAB (diaminobenzidine) dye and counterstained with methyl green. Slides were observed under light microscope for apoptosis detection. Dark brown stained cells were marked as apoptotic in comparison with light brown live cells.

RESULTS AND DISCUSSION

IR Spectra: The infrared spectral data of the ligand and their metal complexes are presented in table 1. IR band at 1612 cm^{-1} due to –CH=N shifted to lower values by 15-30 cm^{-1} in the spectra of metal complexes indicating coordination through azomethine nitrogen atom [28]. The IR bands in the range 1443-1466, 1504-1535 and 1041-1042 were assigned respectively to --C=C, -C=N, -N-N of pyrazole ring [29]. A broad band at 2700 cm^{-1} in the ligand due to –SH vibrations disappeared in the IR spectra of complexes suggesting coordination through sulfur [30].

Table 1. Important IR spectral bands (cm^{-1}) of Schiff base and its metal complexes

Compound	ν(N=CH)	ν(C-S)	ν(S-H)	ν($OOCCH_3$)	ν(H_2O/OH)	ν(M-S)	ν(M-N)
Schiff base	1612	-	2700	-	-	-	-
Co(L)(OAc).3H_2O	1592	764	-	1736	3155	342	470
$Co(L)_2$.2H_2O	1586	741	-	-	3155	318	468
Ni(L)(OAc).3H_2O	1597	756	-	1744	3155	318	470
$Ni(L)_2$.2H_2O	1582	764	-	-	3155	342	470
Cu(L)(OAc)H_2O	1597	754	-	1774	3310	318	468
$Cu(L)_2$	1602	738	-	-	-	318	470
n(L)(OAc).3H_2O	1590	734	-	1744	3148	342	470
$Zn(L)_2$.2H_2O	1593	725	-	-	3148	342	468

^{1}H NMR Spectra: The ^{1}H NMR Spectra of Schiff base and its Zn (II) complexes were recorded in DMSO-d_6 at room temperature (Table 2). The spectrum of the ligand displays azomethine proton (-CH=N-) as a singlet at 8.71 ppm which shifted to downfield region in the spectra of the Zn (II) complexes suggesting that the azomethine nitrogen atom is taking part in complexation with the metal ion [31]. The signal for the SH proton appeared at 14.1 ppm in the spectrum of the free ligand; this signal disappeared in the spectra of its Zn(II) complexes indicating that the Schiff base is coordinated to the metal ions by sulphur atom [30].

Table 2. ^{1}H NMR spectral data of Schiff base and its Zn(II) complexes

Compounds	^{1}H NMR (ppm)
Schiff base	7.59 (m, 5H, Ar-H), 8.20 (d, 2H, Ar-H), 8.29 (d, 2H, Ar-H), 2.15 (s, 3H, triazine-CH_3), 9.28 (s, 1H, pyrazole-H), 8.71 (s, 1H, -N=CH-), 14.1 (s, 1H, -SH)
Zn(L)(OAc).3H_2O	7.56 (m, 5H, Ar-H), 8.27 (d, 2H, Ar-H), 8.16 (d, 2H, Ar-H), 2.14 (s, 3H, triazine-CH_3), 9.13 (s, 1H, pyrazole-H), 8.86 (s, 1H, -N=CH).
$Zn(L)_2$ .2H_2O	7.57 (m, 10H, Ar-H), 8.26 (d, 4H, Ar-H), 8.16 (d, 4H, Ar-H), 2.14 (s, 6H, triazine-CH_3), 9.11 (s, 2H, pyrazole-H), 8.84 (s, 2H, -N=CH)

Fluorescent characteristics: The ligand exhibits small fluorescent peak at 463 nm. It is interesting that the complexes show a higher intensity than that of the free ligand with emission peaks at 447 nm for Co(II), 471 nm for Ni(II), 486 for Cu(II) and 370 nm for Zn(II) complexes (Figure 2). The increase in fluorescence intensity of the complexes may be due to the factor of chelation induced rigidity and a restriction in the PET process [32].

Thermal Analysis: Thermogravimetric analysis of all the complexes were carried out in air atmosphere at a heating rate of 10°Cmin^{-1} from ambient to 800°C. All the complexes decomposed in two to three steps

(Figure 3) leaving metal oxide as residue. Hence, TG and DTA curves of $Co(L)_2.2H_2O$ and $Cu(L)(OAc).H_2O$ have been presented. The data are provided in table 3. From the TG curves it is observed that the first step corresponds to loss of water molecules and the subsequent steps show the decomposition of organic moiety and triazine ring respectively.

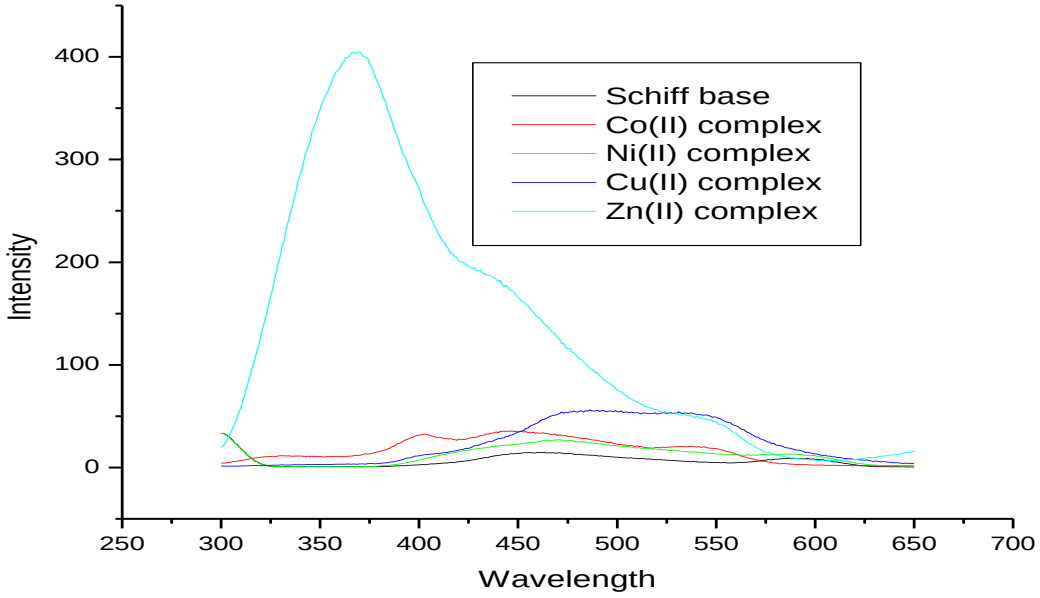


Figure 2. Fluorescence spectra of Schiff base and its 1:2 metal complexes

Table 3. Thermogravimetric data of metal complexes

Compound	Temp (°C)	Decomposed moiety	Mass %		Residue %		Nature
			Calcd.	Found	Calcd	Found	
$Co(L)_2.2H_2O$	130-230	H_2O	3.75	3.81	7.81	7.84	CoO
	230-380	Organic moiety	57.70	57.20			
	380-540	Triazine	30.61	30.64			
$Cu(L)(OAc).H_2O$	130-240	H_2O	3.14	3.11	13.80	12.97	CuO
	241-360	Organic moiety	58.80	58.10			
	361-450	Triazine	24.2	24.70			

ESR spectra: The 1:1 and 1:2 Cu(II) complexes possess a characteristic ESR spectrum with two g values, $g_{\parallel}$ and $g_{\perp}$. For $Cu(L)OAc.H_2O$, $g_{\parallel} = 2.23$, $g_{\perp} = 2.14$, $g_{av} = 2.17$ and for $Cu(L)_2$, $g_{\parallel} = 2.12$, $g_{\perp} = 2.04$, $g_{av} = 2.06$. The trend $g_{\parallel} > g_{\perp} > 2.0023$ indicates the presence of unpaired electron in $d_{x^2-y^2}$ orbital of the Cu(II) ion [33]. For the 1:1 and 1:2 complexes, the G values are 2.65 and 3.1 suggesting that there is significant exchange coupling in the solid complexes.

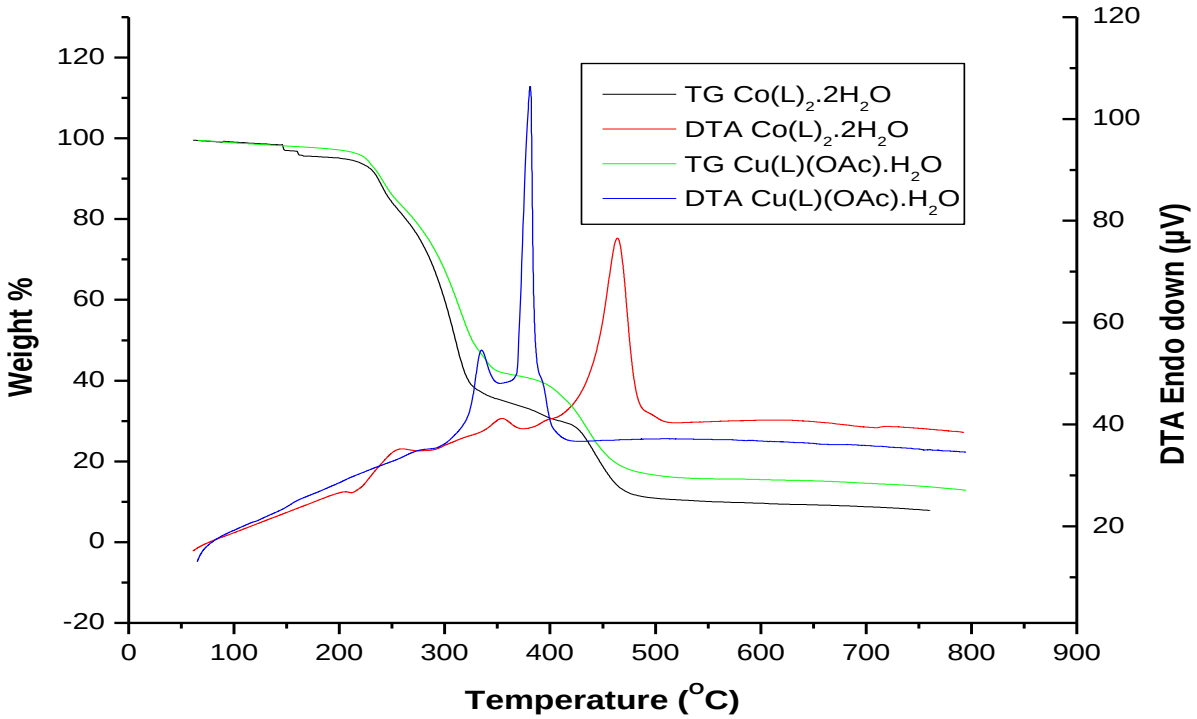


Figure 3. TG and DTA curves of metal complexes

Apoptosis study: The *in vitro* studies for the detection of apoptosis revealed the nature of synthesized compounds as apoptotic inducer. The Histo-morphological analysis demonstrated the induction of degenerative changes in the testicular tissue and its germ cell post treatment with the synthesized compounds in comparison with control displayed normal cell morphology (Figure 4a) with well-defined cell-cell association, intact cellular membrane and uniformly stained nucleus. Whereas apoptosis characteristics were manifested histologically by the listed compounds (Figure 4b-j; 400X, 1000X) revealing increased vacuolization, pyknotic nuclei with highly condensed and densely stained chromatin, crescent shaped chromatin (Figure 4e; $Ni(L)_2.2H_2O$) and presence of empty spaces within cell (Fig. 4b-j inset; 1000X). Automated fluorescence assay was done for recognition and quantification of apoptosis using acridine orange that showed red fluorescence in the live normal cells with intact cell membrane and normal nucleus while apoptotic cell fluorescents bright green (Figure 5). In testicular germ cells treated with newly synthesized ligand and metal complexes revealed presence of increased apoptosis in comparison with control (Figure 5) that is shown with the high incidence of green fluorescence in the treated groups. $Co(L)(OAc).3H_2O$ and $Cu(L)_2$ were found to be most potent apoptosis inducer with percentage apoptosis 55 ± 4.1 and 39.41 ± 1.39 respectively followed by $Co(L)_2.2H_2O$ (39 ± 2.08) and $Ni(L)(OAc).3H_2O$ (34.33 ± 0.88) in comparison with control (4.33 ± 0.88) (Figure 5a-j). When $Zn(L)_2.2H_2O$ was shown to possess high apoptotic activity (31.33 ± 0.88), $Ni(L)_2.2H_2O$ was least apoptotic among all compounds with 26.33 ± 0.33 percentage apoptosis followed by Schiff base, $Cu(L)(OAc)H_2O$ and $Zn(L)(OAc).3H_2O$ with 28 ± 2.00, 28.33 ± 1.85 and 28.66 ± 2.02 percentage apoptosis respectively (Figure 5e-f, h, j). DNA fragmentation was done with the help of *in situ* end labeling (TUNEL) assay using DAB stain with methyl green as counter strain. The microphotograph of testicular sections (Figure 6) showed the nature of ligand and the metal complexes as apoptotic with dark brown cells indicating fragmented DNA. In $Co(L)(OAc).3H_2O$ and $Cu(L)_2$ (Figure 6b, g; 400X), increased incidence of DNA fragmentation was observed as compared to control (Figure 6a; 1000X). It was observed that $Ni(L)_2.2H_2O$ treatment showed least DNA fragmentation (Figure 6e; 400X). $Cu(L)(OAc)H_2O$, $Zn(L)(OAc).3H_2O$ and Schiff base showed almost similar results (Figure 6f, h, j; 1000X).

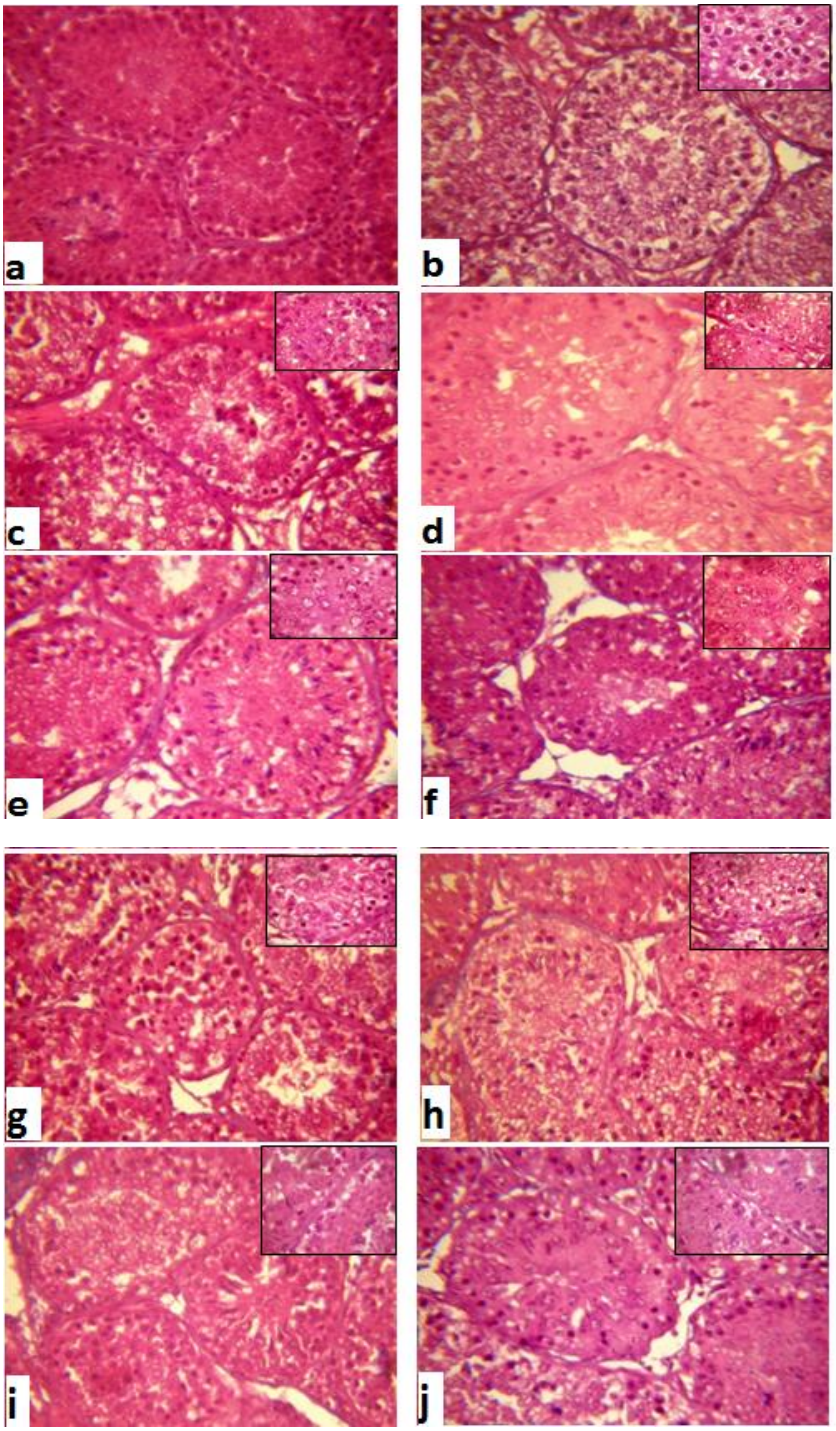


Figure 4. Light micrograph of testicular sections showing normal well defined cellular associations and stages in control (a) and presence of apoptotic characteristics of testicular germ cells along with degenerative changes in testicular sections treated with compounds (b-j) at 400X (inset 1000X) post hematoxylin and eosin staining. Treated testicular sections are characterized by increased vacuolization, germ cells with crescent shaped nucleus (arrowhead), condensed pyknotic nuclei and degenerative luminal spaces (b-j).

a)Control **b)** $Co(L)(OAc).3H_2O$ **c)** $Co(L)_2.2H_2O$ **d)** $Ni(L)(OAc).3H_2O$ **e)** $Ni(L)_2.2H_2O$
f) $Cu(L)(OAc).H_2O$ **g)** $Cu(L)_2$ **h)** $Zn(L)(OAc).3H_2O$ **i)** $Zn(L)_2.2H_2O$ **j)** Schiff base

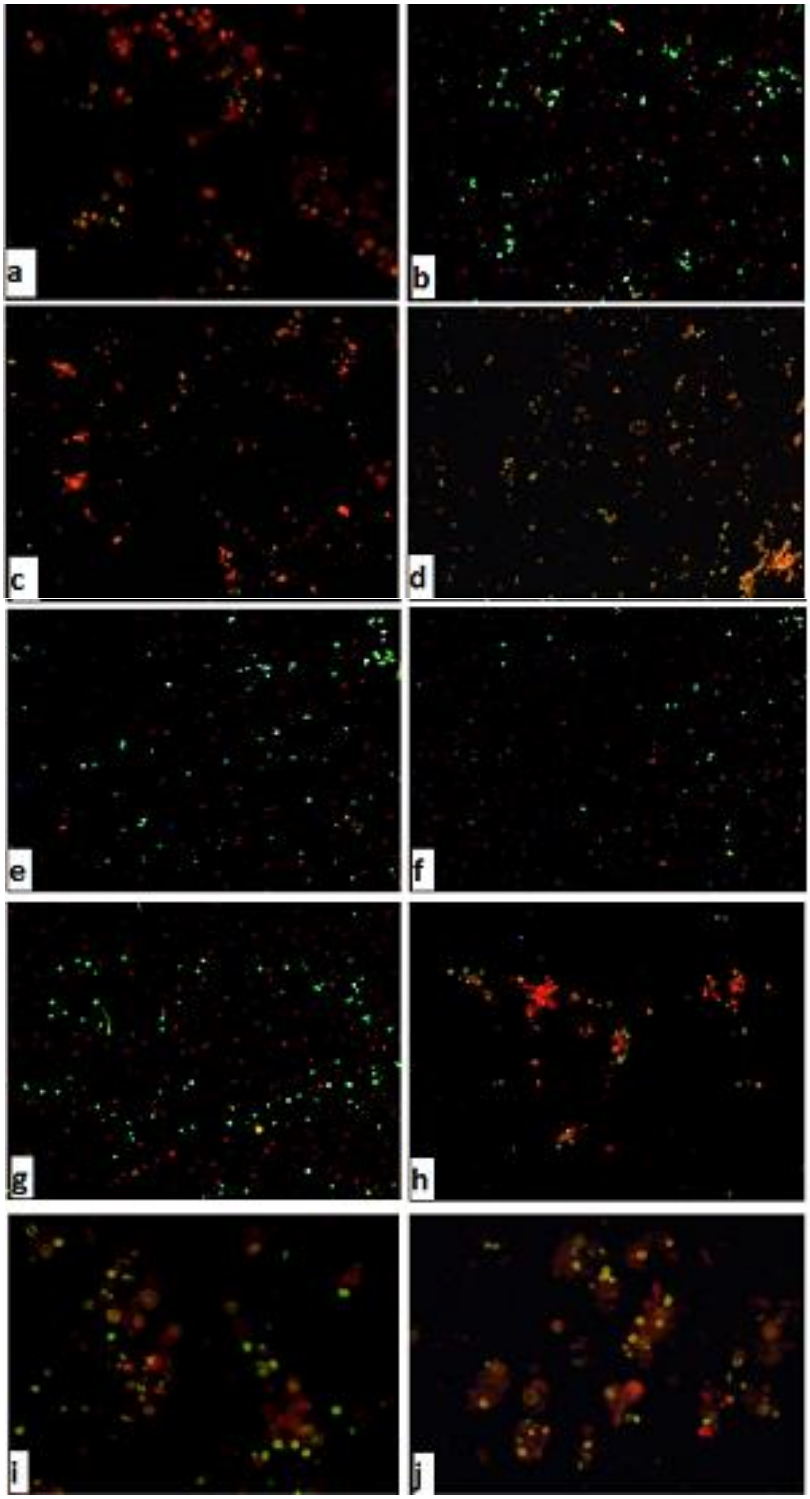


Figure 5 Fluorescent photographs of testicular germ cells treated with compounds (b-j) showing apoptotic germ cells with bright green fluorescence and normal live germ cells with red fluorescence at 100X (a-h) and 400X (i-j).

a) Control **b)** $Co(L)(OAc).3H_2O$ **c)** $Co(L)_2.2H_2O$ **d)** $Ni(L)(OAc).3H_2O$ **e)** $Ni(L)_2.2H_2O$
f) $Cu(L)(OAc).H_2O$ **g)** $Cu(L)_2$ **h)** $Zn(L)(OAc).3H_2O$ **i)** $Zn(L)_2.2H_2O$ **j)** Schiff base

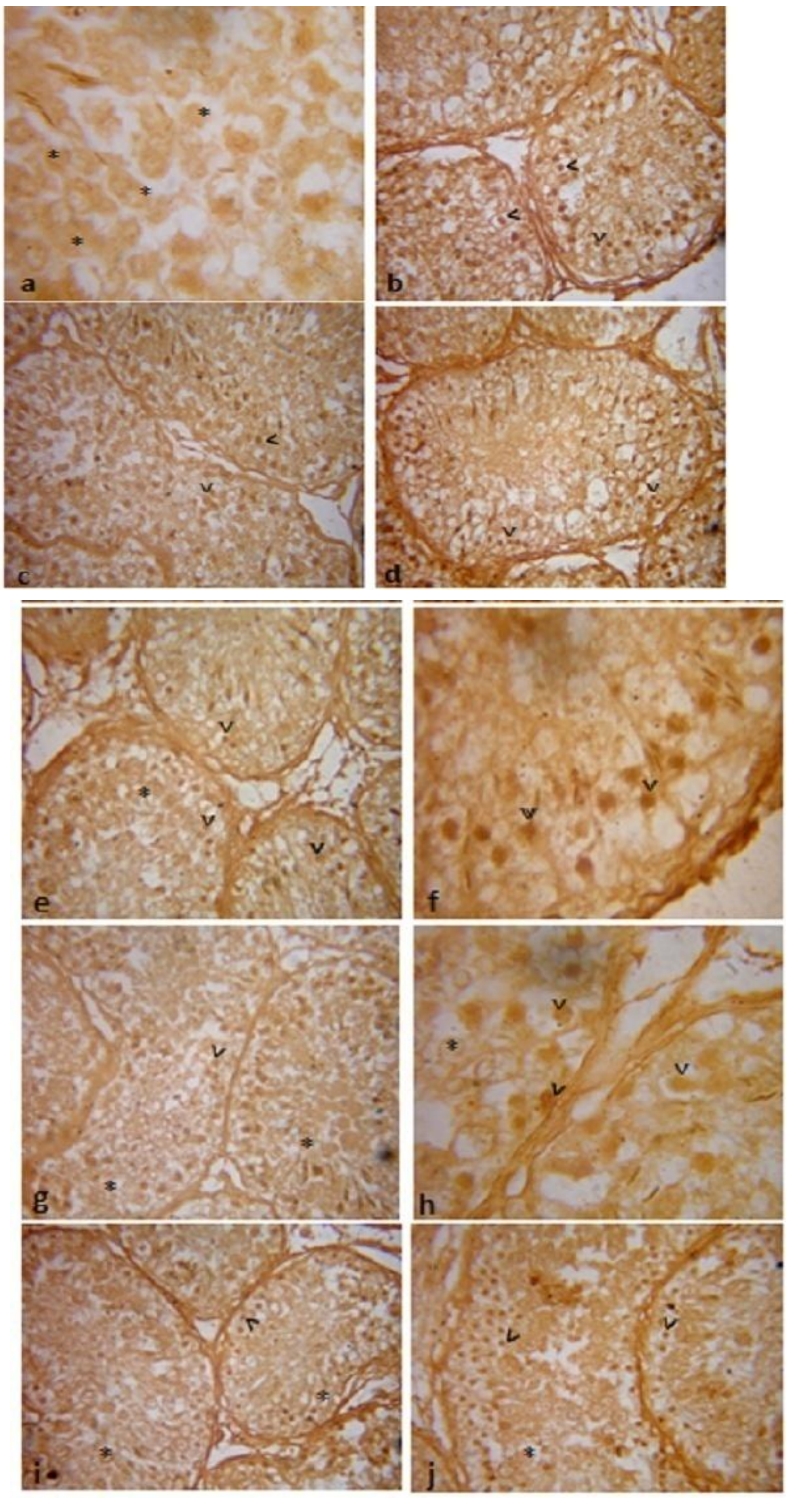


Figure 6 Microphotographs showing apoptosis via TUNEL assay in testicular germ cells after treatment with compounds (b-j) demonstrating dark brown apoptotic germ cells (arrowheads) and normal cells (asterisks) at 400X (b-e, g, i) and 1000X (a, f, h, j).

a) Control **b)** $Co(L)(OAc).3H_2O$ **c)** $Co(L)_2.2H_2O$ **d)** $Ni(L)(OAc).3H_2O$ **e)** $Ni(L)_2.2H_2O$
f) $Cu(L)(OAc)H_2O$ **g)** $Cu(L)_2$ **h)** $Zn(L)(OAc).3H_2O$ **i)** $Zn(L)_2.2H_2O$ **j)** Schiff base

APPLICATIONS

It has been reported that pyrazole derivatives could inhibit cell proliferation and promote cell apoptosis. Tozasertib, also known as VX-680 or MK-0457, a 3-aminopyrazole derivative that inhibits Aurora kinases, induces apoptosis in tumor cells [15]. Therefore, we envisioned the synthesis of Schiff base complexes derived from condensation of pyrazole based aldehyde and substituted 1,2,4-triazine with the objective to obtain high apoptotic activities by combining two different biologically active moieties. Our results illustrate the response of all the compounds to be apoptotically active. In addition, existing literature reveals that the analogs with substituents having electron withdrawing nature show better activity than electron releasing substituents [34]. In our case, the presence of $–NO_2$ group (strongly electron-withdrawing) on the pyrazole ring seems to be responsible for better apoptotic behavior of all the newly synthesized compounds. Further, metal complexes are found to be more active inducers as compared to the ligand indicating that the presence of metal ions may disrupt the mitochondrial membrane potential, inducing the caspase-3-activation and thus leading to induction of apoptosis. The observed apoptotic potential of the complexes is as follows: Co(II) complex > Cu(II) complex > Zn(II) complex > Ni(II) complex.

CONCLUSIONS

In conclusion, we report that all the synthesized compounds are inducers of apoptosis resulting in DNA fragmentation. Results have shown the enhancement of apoptosis activity by chelation and two complexes, i.e., 1: 1 Co complex and 1:2 Cu complex displayed increased incidence of DNA fragmentation in comparison with the control. The present findings support the therapeutic potential of the synthesized metal complexes and may thus attract chemists to study and understand the mechanism of apoptosis in a better way.

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