6,6',8,8'-TETRAMETYL-4,4',7.7'-TETRAHYDROS-7,7'-BI-1,2,4,5-TETRAZOCINE-3,3'(2H,2'H)-DITHIONE, A NOVEL HYDRAZONE SCHIFF BASE WITH PROMISING PHARMACOLOGICAL EFFECTS

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Abstract. Novel hydrazone Schiff base viz. 6,6',8,8'-tetramethyl-4,4',7,7'-tetrahydro-7,7'-bi-1,2,4,5-tetrazocine-3,3'(2*H*,2'*H*)-dithionehas been synthesized from3,4-diacetylhexane-2,5-dione and thiocarbohydrazideandcharacterized. The bicyclichydrazone Schiff basehas been found active against bacteria[*S.aureus* and *K.pneumoniaae*] and fungi [*A.niger* and *Trichophytonrubrum*]. DNA photo-cleavage and antiangiogenic behavior of synthesized hydrazone Schiff base has been studied. Furthermore,the synthesizedhydrazone Schiff basesignificantly inhibited cell proliferation with IC_{50} value 62.5µg/ml. The flow-cytometry analysis of hydrazone Schiff base treated human breast cancer cells showed that apoptosis rate increased with increase in dose. In conclusion present study proposes the development of novel therapeutic strategy for MCF-7 cancer cell treatment.

Keywords: 3,4-diacetylhexane-2,5-dione; thiocarbohydrazide; antimicrobial activities; anti-angiogenesis; DNA-cleavage and anti-proliferation

Introduction

The previous decades showed intensive studies to synthesize ligand using of Schiff's bases due to their flexible chelating capabilities with pharmaceutical activities (Efthimiadou et al.,2007; Sathisha et al., 2008; Gudasi et al., 2008). Sulphur-containing compounds such as thiosemicarbazones, structural analogues of the thiocarbohydrazone, have been reported for potent anticancer activity (Williams,1972; Patil et al.,1989; Klayman et al., 1983; Suarez Iha et al., 1994; Terra et al., 1999). Similarly, presence of azomethine functionality in ligand is known to be responsible for its antibacterial, antifungal, antitumor antituberculosis, anticancer, antihelminthics, DNA binding and DNA cleavage properties (Patai, 1970).

The biological activity of synthesized ligand mainly depends upon its molecular structure which in turn regulated by the type of reacting molecules. Thiocarbohydrazide, a versatile reacting material, has been used widely to construct

a variety of heterocyclic derivatives which are known to possess significant antifungal and antimicrobial properties (Lozana et al., 2003). As previously reported the anticancer activity of a drug might be due to its interaction with cancer cell DNA. Scientific communities are trying hard to explore the DNA cleaving /binding studies of Schiff base ligands and their complexes so as to purpose a novel therapeutic strategy for cancer treatment (Erkkila et al., 1999; Chan&Wong, 1995;Liang et al., 2004).

Also tumor cells are known to induce angiogenesis through the activation of endothelial cells followed by pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor [FGF] and epidermal growth factor [EGF] (Tuli et al., 2014). These factors are highly expressed and associated with growth of various types of human tumors (Breier&Risau, 1996; Ferrara et al., 2003). Therefore, compounds with anti-angiogenic properties have been identified as an attractive strategy for the treatment and prevention of cancer.

In the light of above reports the present work deals with the synthesis, characterization followed by evaluation of antimicrobial, anti-cancer, anti-angiogenic and DNA photo-cleavage activities of novel hydrazone Schiff base ligand.

Experimental

Materials

All the reagent and nutrient medium ingredients of analytical grade and purchased from E-Merck India and Hi media Ltd (India) respectively. Other chemicals including solvents were of LR grade and were purified by standard procedure prior to use. All the reactions were performed on magnetic stirrer equipped with hotplate. Double-distilled water was used in the present study.

Synthesis and characterization of hydrazone Schiff base ligand

Both 3,4-diacetylhexane-2,5-dione(0.1 mol) and thiocarbohydrazide (0.2 mol) were prepared as reported earlier (Kurzer &Wilkinson, 1970) and mixed in a mortar pestle followed by 06h refluxing with continuous stirring using ethanol as solvent. The appeared solid was collected by filtration and dried over anhydrous $CaCl_2$ in vacuum desiccator. Purity of the compound was initially evaluated by TLC (as single spot) on silica gel-G developed in anhydrous methanol and THF (1:3) solvent. Elemental (C H N) analysis was performed on a Perkin Elmer 2400 CHN elemental analyzer. H^1NMR spectra of ligand in DMSO solution was recorded on Bruker Avance II 400MHz Spectrometer and chemical shifts were indicated in ppm relative to tetramethylsilane. IR spectra were recorded in KBr disks on a Perkin-Elmer FTIR 31725X spectrophotometer. Mass spectra were measured in the range of m/*z* = 200-1400. All these spectroscopic studies were carried out at SAIF lab, Punjab University, Chandigarh, India.

6,6',8,8'-tetramethyl-4,4',7,7'-tetrahydro-7,7'-bi-1,2,4,5-tetrazocine-3,3'(2H,2'H)-dithione

Light yellowish solid, Yield: 63% M.p:270^{0}C, IR (KBr,cm^{-1}) 1633(s), ν(>C=N); 3233(s), ν(N-H); 1243(s), ν(>C=S). ^{1}H NMR (DMSO-d_6, 400MHz) (ppm): 1.07-1.10 (CH_3); 1.98 (CH); 2.5 (NH). MS: m/z $[M]^+$338.4. Anal.calcd. for $C_{12}H_{18}N_8S_2$: C 41.9, H 5.4 , N 32.9, found C 42.6, H 5.3 , N 33.1.

Disc diffusion assay

In vitro antimicrobial activity has been evaluated for synthesized ligand against pathogenic strains of bacteria (*Staphylococcus aureus*, *Klebsiellapneumoniae*) and fungi (*Aspergillusniger*, *Trichophytonrubrum*) using disc plate diffusion assay (Kisangau et al., 2007). The stock solutions of ligand (1000 ppm) was prepared in DMSO. The plates of culture medium using nutrient agar and potato dextrose agar were prepared for bacterial and fungal growth respectively under sterilized condition. The various concentrations of ligand (100, 150, 200 and 250 ppm) were loaded on 5 mm sterilized filter paper discs and placed on agar plates followed by incubation at 30°C for 24 h and 72 h to evaluate the effect of compound on bacterial and fungal growth respectively. Neomycin and Fluconazole were used as standard antimicrobial agents for bacterial and fungal study respectively.

Chorioallantoic membrane (CAM) assay

Anti-angiogenic activity has been evaluated using *ex-vivo* CAM assay (Ribatti et al., 1996). The fertilized chicken eggs were collected, cleaned with 70% ethanol to avoid infections and kept in a humidified (70%) chamber at 37°C. After 48 h, 1 ml of albumin was taken out with a syringe from the lower side of the eggs and the pierced holes were sealed with a sterilized laboratory tape. After 72h of incubation, a small window was made by removing the egg shell at the blunt end. On confirmation of normal and viable development of the embryo, various concentrations of synthesized ligand (0, 1 and 10 µg) were loaded on 5 mm sterilized filter discs and placed over the surface of extra embryonic membrane i.e. CAM. The windows were sealed with sterilized laboratory tape to prevent external environmental contact and eggs were kept for 48h under incubation. After the treatment, anti angiogenic effect of ligand was manually counted in terms of branch points over CAM and calculated the percent inhibition as follows:

$$\%\text{Inhibition} = \text{Data of control} - \text{Data of treated} \times 100 / \text{Data of control}$$

DNA photo-cleavage assay

DNA cleavage activity of the synthesized ligands were studied by agarose gel electrophoresis using supercoiled pUC19 plasmid DNA (Pal et al., 2014; Rani et al., 2015). The total volume of reaction mixture was 10 µl containing 0.5 µg of

plasmid DNA in TE (*Tris* 10mM, EDTA 0.01mM, pH 8.0) buffer with various concentrations of synthesized ligands. The eppendorfs carrying reaction mixture were placed directly on the surface of a trans-illuminator (8000 mW/cm), at 360 nm for 30 min. After irradiation, samples were further incubated at 37°C for 1 h. Irradiated samples were mixed with 6X loading dye containing 0.25% bromophenol blue and 30% glycerol. The samples were then analyzed by electrophoresis on a 0.8% agarose horizontal slab gel in *Tris*-Acetate EDTA buffer (40 mMTris, 20 mM acetic acid, 1 mM EDTA, pH: 8.0) with comparison to untreated plasmid DNA as a control. Gel was stained with ethidium bromide (1 µg/ml) and photographed under UV light.

Cytotoxicity assay

MCF-7 cells were maintained in T-25 tissue culture grade flask in DMEM medium supplemented with 10% FCS, 3.7 g/l sodium bicarbonate, 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C under 5% CO_2 / 95% air in humid environment. The cells were used for the experiment at 70% to 80% confluences.

MTT assay was performed on MCF-7 cells to determine the viability of cells exposed to different concentrations (31.5 µg/ml to 1000 µg/ml) of ligand. Treated and untreated cells were incubated in 96 wells plate for 24 h at 37°C under 5% CO_2 in an incubator. MTT solution 10 µl (5 mg/ml) was added to each well and mixed by tapping gently on the side of the tray and further incubated for 4 h. After the incubation, the purple crystals were observed which were dissolved by adding 100 µl DMSO into each well. Optical density was measured at 570 nm using Multiplate reader (Perkin Elmer) and percent (%) viability was calculated as follows:

Percent (%) Viability = 100 x Mean of Test O.D. / Mean of Control O.D.
Percent (%) Cytotoxicity = 100 – (Percent viability)

Cell cycle analysis

MCF-7 cancer cells (50×10^4) were seeded in 24 well plates with complete DMEM medium., Cells were treated with medium containing various concentrations of ligand (125, 200, 250, 500 µg/ml). After incubation for 24 hours, cells were harvested, washed twice with phosphate buffered saline (PBS), and then fixed by -20° C chilled 70% ethanol for overnight. Thereafter, cells were re-suspended in 400 µL PBS and added with 50 µl of PropidiumIodidie (PI) solution (400 µg/ml) and 50 µL of RNase solution (1mg/ml) followed by 30 min incubation at 37 °C under dark condition. Cell cycle analysis was done on BD FACSCalibur™ flow cytometer using BD CellQuest ™ after gating out doublet in PI area vs width plot. Cell fractions in different phases of cell cycle were determined using Cyflogic™ (Cyflo Ltd, Finland) analysis software.

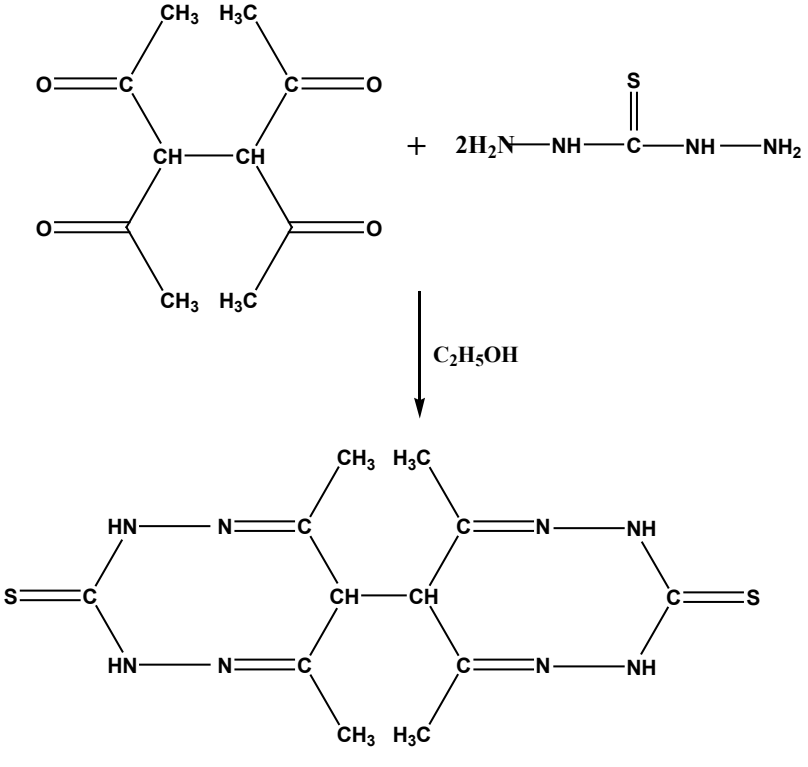
Detection of apoptosis using Annexing V- Alexafluor 488

5x10^{4}cells were cultured in 24 well plates for overnight followed by treatment of ligand at concentrations of 125, 200, 250 and 500 µg/ml. After 24 h of treatment, cells were harvested, washed and re-suspended in 100 µL Annexin-V binding buffer. Cells were stained with 5 µl of anti-Annexin-V-Alexafluor 488 (Molecular Probes) and PI according to manufacturer's protocol. The results were analysed on BD FACSCalibur™ equipped with Cyflogic™ (Cyflo Ltd, Finland) software.

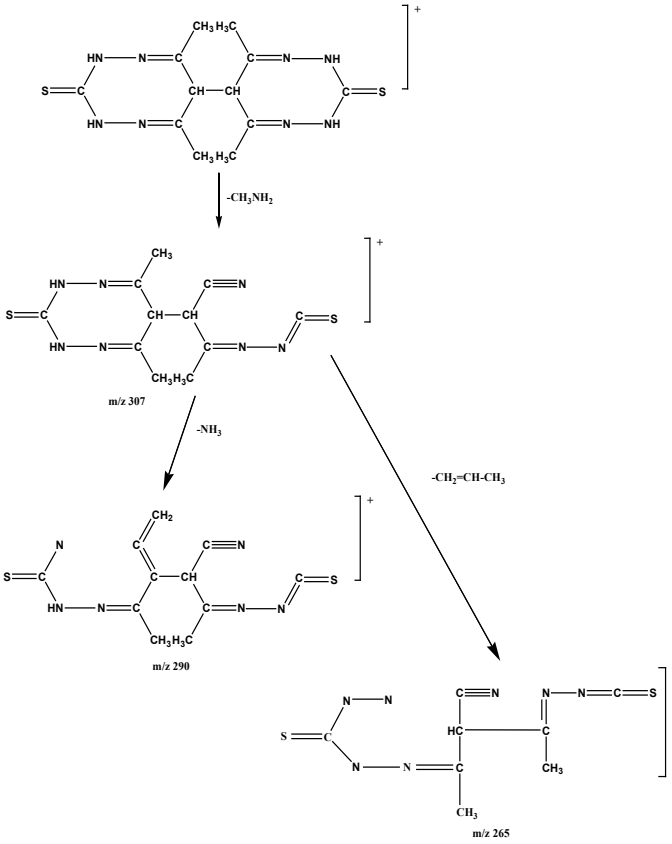
Results and discussion

Chemistry

The hydrazone Schiff base was obtained after refluxing with continuous stirring of3,4-diacetylhexane-2,5-dion (1 mole) and thiocarbohydrazide (2 moles) in ethanol (Scheme 1). Melting point of hydrazone Schiff base (Table 1) was 270°C. The synthesized hydrazone Schiff base found soluble in DMSO and DMF.



Scheme 1. Synthesis of hydrazone Schiff base (6,6',8,8'-tetramethyl-4,4',7,7'-tetrahydro-7,7'-bi-1,2,4,5-tetrazocine-3,3'(2*H*,2'*H*)-dithione)



Scheme 2. Proposed mass fragmentation pattern of synthesized hydrazone Schiff base

Table 1. Physical and analytical data of hydrazone Schiff base

	Molecular formula	Melting point (°C)	Found(Calc.)% C	H	N	Molecular mass Found(Calc.)
Hydrazone Schiff base	$C_{12}H_{18}N_8S_2$	270	41.9 (42.6)	5.4 (5.3)	32.9 (33.1)	338.4 (338)

The IR spectra of starting materials and hydrazone Schiff base have been recorded. Their comparative study confirmed the formation of proposed hydrazone Schiff base. The IR Spectra of 3,4-diacetylhexane-2,5-dione showed a strong absorption band at 1600cm^{-1} for carbonyl groups present although the characteristics absorption of ketonic group is 1680-1750cm^{-1}. The reason of absorption by 3,4-diacetylhexane-2,5-dioneat

1600 cm^{-1} is strong intra/inter-molecular hydrogen bonding. The absorption bands present in the spectra of 3,4-diacetylhexane-2,5-dioneand thiocarbohydrazide at 1600 cm^{-1} and 3475 cm^{-1} due to ν (>C=O) and ν(-NH_2) groups were found absent in the spectra of hydrazone Schiff base and a new sharp and strong band at 1633 cm^{-1} appeared which clearly indicated that condensation indeed occurred and was attributed to ν(>C=N) (Chiswell,& O'Reilly, 1979; Chiswell et al., 1980).

Another band in the spectra of hydrazone Schiff base at 1243 cm^{-1} was assigned to >C=S stretching. The absorption at 1501cm^{-1} has given N-H bending vibration. The N-H stretching absorption appeared at 3233cm^{-1} in the spectra of hydrazone Schiff base.

The H^1 NMR spectra of hydrazone Schiff base showed a singlet at δ 1.07-1.10 ppm assigned to methyl protons, a doublet at δ 1.98 ppm attributed to C-H protons, while N-H protons were shown by a singlet at δ 2.5 ppm. The mass spectra of hydrazone Schiff base confirmed the probable formula by showing molecular ion peak at 338.4 (m/z) following the nitrogen rule and corresponding to bicyclic thiocarbohydrazone moiety. Scheme 2 represents the proposed mass fragmentation pattern for major peaks found in spectra.

Antimicrobial activities

The inhibitory effects of synthesized hydrazone Schiff base towards harmful human pathogenic microorganisms were evaluated with comparison to Neomycin and Fluconazole. The hydrazone Schiff base was found very potent against tested bacteria and fungi (Fig.1). The antimicrobial action of hydrazone Schiff base was measured in term of zone of inhibition (Table 2) and MIC values were found to be in the range of 85-100 µg/ml.

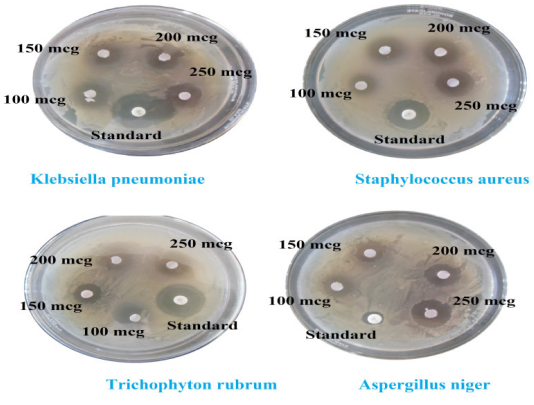


Figure 1. Photograph showed antimicrobial activities of synthesized Schiff base

Anti-angiogenesis activity

The chicken chorioallantoic membrane (CAM) is a simple and commonly used model for the initial screening of potential inhibitors of angiogenesis.

Table 2. Antimicrobial result of Hydrazone Schiff base hydrazone Schiff base. Data represented as the average mean of three values with ±0.5 to ±0.7 standard deviation

Hydrazone Schiff base		Bacterial Strains (Zone of inhibition in mm)								Fungal Strains (Zone of inhibition in mm)							
		S. aureus				K. pneumoniaae				A. niger				Trichophytonrubrum			
Dosages	100 ppm	150 ppm	200 ppm	250 ppm		100 ppm	150 ppm	200 ppm	250 ppm	100 ppm	150 ppm	200 ppm	250 ppm	100 ppm	150 ppm	200 ppm	250 ppm
Zone of Inhibition	10 mm ±0.5	15 mm ±0.7	17 mm ±0.6		18 mm ±0.6	11 mm ±0.5	12 mm ±0.5	15 mm ±0.5	18 mm ±0.7	12 mm ±0.5	15 mm ±0.5	17 mm ±0.5	20 mm ±0.5	11 mm ±0.7	13 mm ±0.4	14 mm ±0.5	16 mm ±0.6
Standards	21 mm (Neomycin , 250 ppm)					23 mm (Neomycin, 250 ppm)				24 mm (Fluconazole, 250 ppm)				9 mm (Fluconazole ,250 ppm)			

The ability of synthesized hydrazone Schiff base to inhibit the blood vessels on CAM was observed as shown in Fig.2. Percentage of inhibition of vessel growth rate in hydrazone Schiff base treated groups were 29% (1 µg), and 90% (10 µg) in comparison with control. The results revealed that synthesized hydrazone Schiff base hydrazone Schiff base has potential to stop the growth of neo-vascularization on CAM.

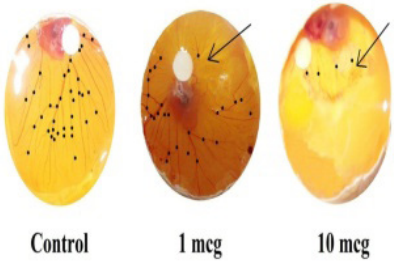


Figure 2. The photograph showed

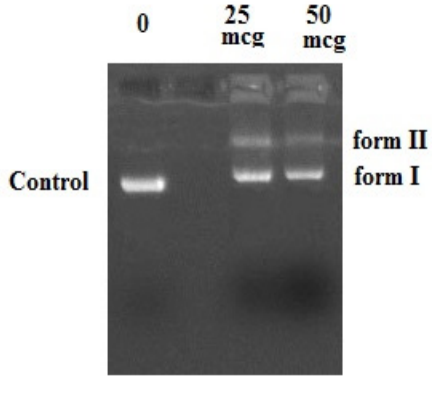


Figure 3. Cleavage of supercoiled pUC19 DNA by synthesized hydrazone Schiff base

DNA photo-cleavage assay

The photo-induced DNA cleavage has been carried out using super coiled plasmid DNA by gel electrophoresis. The super coiled DNA (form I) migrate relatively fast with comparison to nicked DNA (form II). The conversion of form I to form II was observed with the treatment of synthesized hydrazone Schiff base in comparison to untreated plasmid DNA (Fig. 3) which indicates that hydrazone Schiff base has significant DNA-cleavage potential.

Hydrazone Schiff base inhibited cell growth of MCF-7 cells

The effect of synthesized compound on cell growth and viability in MCF-7 cell lines was observed using MTT assay (Tuli et al., 2015). After 48 hours of treatment with various concentrations of synthesized hydrazone Schiff base, MCF-7 cells showed a significant dose-dependent decrease in the number of metabolically active viable cells (Fig. 4a). Viability of MCF-7 cells was reduced by~28% with 24 h exposure to 125 µg/ml synthesized compound with comparison to DMSO-treated control cells. The morphology of hydrazone Schiff base treated cells was also observed by phase contrast microscope (Fig. 4b). The results imply that synthesized hydrazone Schiff base inhibited the proliferation of MCF-7 human breast cancer cells.

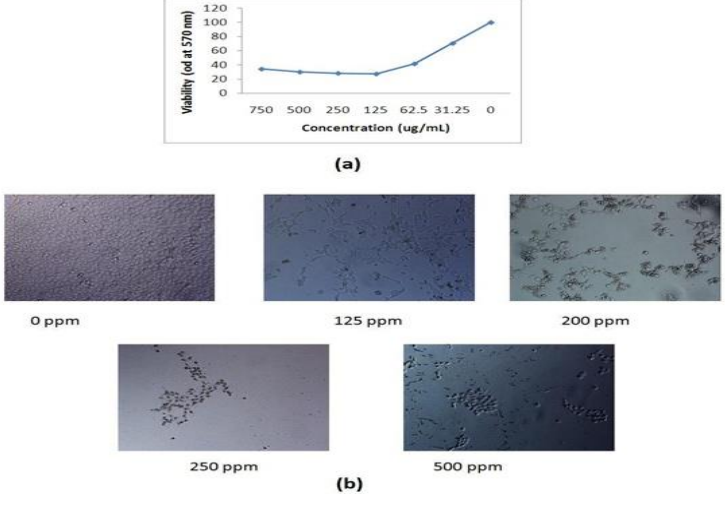
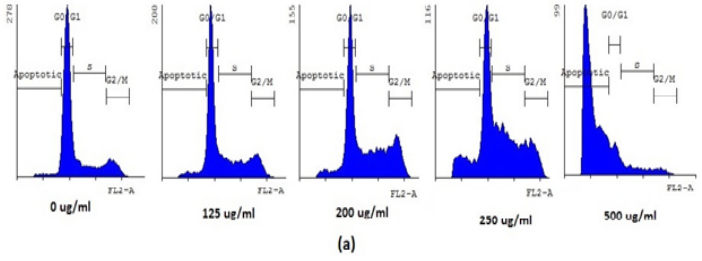


Figure 4. (a) Effect of hydrazone Schiff base (0 to 500 µg/ml) on MCF-7 cells viability was studied using MTT assay and data represented as the mean of three independent experiments. All the experiments were conducted in triplicates and data represented as the average of three replicate with standard deviation. (b) The phase contrast microscopic observation of hydrazone Schiff base treated and untreated MCF-7 cells

Flow cytometric analysis

Growth inhibitory effect of hydrazone Schiff base shown in MTT assay indicated us to assess the cell cycle distribution and subsequently cell death. Based on results of MTT assay, we selected 500, 250 and 125 ppm concentration levels to evaluate cell cycle distribution and cell death using PI staining and Annexin-V/PI respectively. After 24h of treatment, S phase distribution of cells was increased with concomitant decrease in G_0/G_1 fraction. Moreover, proportion of cells in SubG1 region increased with increasing concentration and this decrease in G_0/G_1 phase accompanied by increase in subG1 region indicates growth inhibition and cytotoxic behavior of compound (Fig.5).



(a)

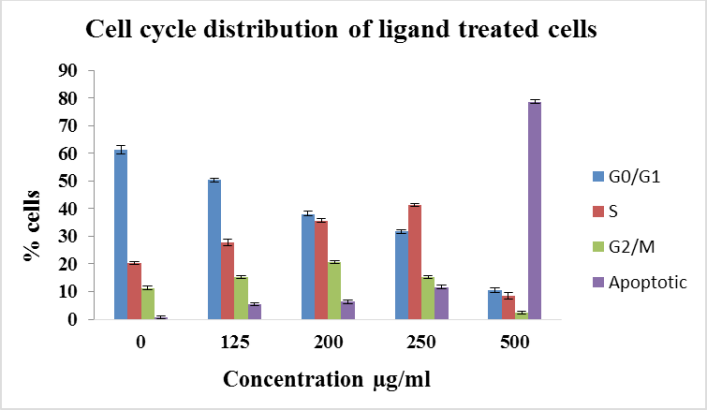


Figure 5. Apoptotic effect of hydrazone Schiff base in MCF-7 cells determined by flowcytometer assay. (a) Representation of flow cytometric plots at various concentrations (0, 125, 200, 250 and 500 µg/ml) of hydrazone Schiff base after 24 h of treatments. (b) The histograms represent cells from the untreated cultures (0 µg/ml) and from the cultures treated with the hydrazone Schiff base that affects the cell cycle distribution and induce apoptosis. All the experiments were conducted in triplicates and data represented as the average of three replicate with standard deviation error bars

Annexin V/PI staining of MCF-7 cells

To further validate the apoptotic observations, treated cells were stained with Annexin-V/PI and analyzed by flowcytometry. AnnexinV-PI staining revealed that hydrazone Schiff base (125-500 µg/mL) significantly inhibited the cells growth in a dose dependent manner. In figure 6, a plot between Annexin-V and Propidium Iodide, from the gated cells showed percentage of viable and non-apoptotic (Annexin-V^-PI^-), early (Annexin-V^+PI^-), and late (AnnexinV^+PI^+) apoptotic cells at various concentration. We found hydrazone Schiff base was efficacious to induce apoptosis even at 125 µg/ml concentration in MCF-7 human breast cancer cells. Percent of live cell was higher in untreated cells and it was decreased with concomitant increase in apoptotic cells with treatment addition and further increase in concentration. The graph also interprets the same result indicating a high percentage of MCF-7 cell death with increase in concentration of hydrazone Schiff base.

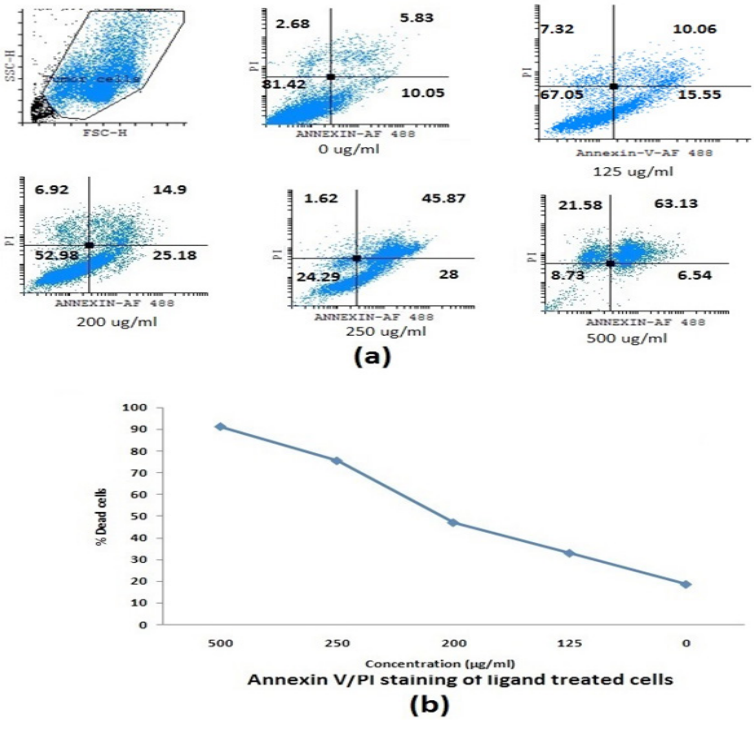


Figure 6. (a) MCF-7 cells were treated with hydrazone Schiff base at the concentration of 0, 125, 200, 250 and 500 µg/ml for 24h and examined using annexin V/propidium iodide staing on flowcytometer. (b) A graph, concentration Vs percentage of dead cells in hydrazone Schiff base treated annexin V/PI stained MCF-7 cells

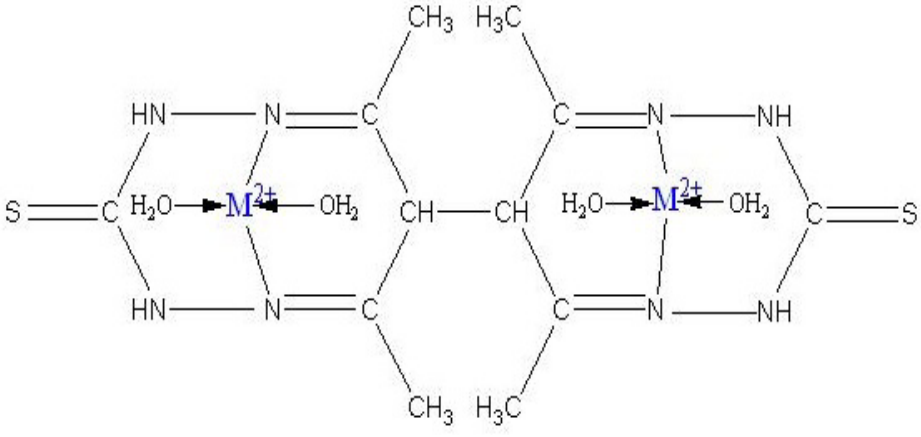


Figure 7. Proposed metal complexes of ligand which could be formed with various transition metals ion (M^{2+} = Zn^{2+}/ Fe^{2+}/ Ni^{2+}/ Cu^{2+} etc.).

Conclusion and future prospective

The present work provides an excellent route for the synthesis of bicyclic thiocarbohydrazone moiety which is found to be biologically active compound. IR, NMR and Mass spectral studies confirm the structure and thus the formation of this novel compound. In addition, this protocol is easy to reproduce. The synthesis method employed in the study holds the potential to be used for production of hydrazone Schiff base in bulk without any side product. The potency of synthesized hydrazone Schiff base was evaluated by antimicrobial and cytotoxicity assay with IC_{50} value 60-100µg/ml. Hydrazone Schiff base inhibits the cell cycle progression and leads to accumulation of cells in SubG1 phase which results in apoptosis induction. Furthermore, It has significant DNA-cleavage and antiangiogenic activity. We hope this study will provide insight to propose the novel therapeutic strategy for infectious and breast cancer treatment.

Furthermore, the structure of ligand suggests that it has azomethine nitrogen atoms which one can imagine could form transition metal complexes in the form of di-, tri- and tetra-dentate ligands as metals can accommodate donor atom's lone pair of electrons into their empty d orbital (Fig.7). Complexity of the resulting compound and its molecular mass can be predicted with the help of spectroscopic tools like UV, IR and mass spectroscopy, respectively, which can further improve the bioactivity of the compounds.

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