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Research Article

Chemistry for New drug discovery

In Vitro Anti-Cancer Activity of Schiff Base 2,4-Dichloro-6-(P-Tolylimino-Methyl)-Phenol and Its Transition metal complexes.

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Abstract: Cancer is the world's second-biggest cause of mortality, accounting for 9.6 million deaths in 2018, and the burden continues to rise. As a result, better newer medications with greater efficacy for the treatment of various tumours are clearly and urgently needed. Anticancer drugs have been grouped as chemotherapy, hormonal remedy and immunotherapy. Chemotherapy protected various categories of drugs, each defined by its chemical structure and mode of action, such as alkylating agents, antibiotics, antimetabolites, mitotic inhibitors, and others. Although this issue has been widely explored, Schiff bases, which are formed from the condensation reaction of aromatic/aliphatic aldehydes and amines and form stable complexes with various transition metal ions, are still of significant interest in inorganic chemistry. Schiff bases and their metal complexes are promising leads for both synthetic and structural research due to their relatively simple synthesis and structural diversity and have been widely investigated, due to their incredible chemical properties and applications in various areas. The chelating ability and biological applications of metal complexes have attracted remarkable attention and they can work as models for biologically important species. Schiff bases and their complexes are flexible compounds synthesized from the condensation of an amino compound with carbonyl compounds and extensively used for industrial purposes and also show a broad range of biological activities including antibacterial, antifungal, antiviral, antimalarial, antiproliferative, anti-inflammatory, anticancer, anti-HIV, anthelmintic and antipyretic properties. The purpose of this research work was to assess the *in vitro* anticancer activity of the synthesized Schiff base molecule and its transition metal complexes against Human breast carcinoma Cancer cells at various doses (1000, 300, 100, 30, 10, and 3 g/ml). The anticancer activity of the free ligand and its metal complexes showed good to high activity against human breast cancer cells. Schiff base and its transition metal complexes against human breast cancer cell lines fascinate the researchers to develop new anticancer drugs without side effects.

Keywords: Schiff base, Metal Complexes, Spectral Characterizations, Anticancer Activity.

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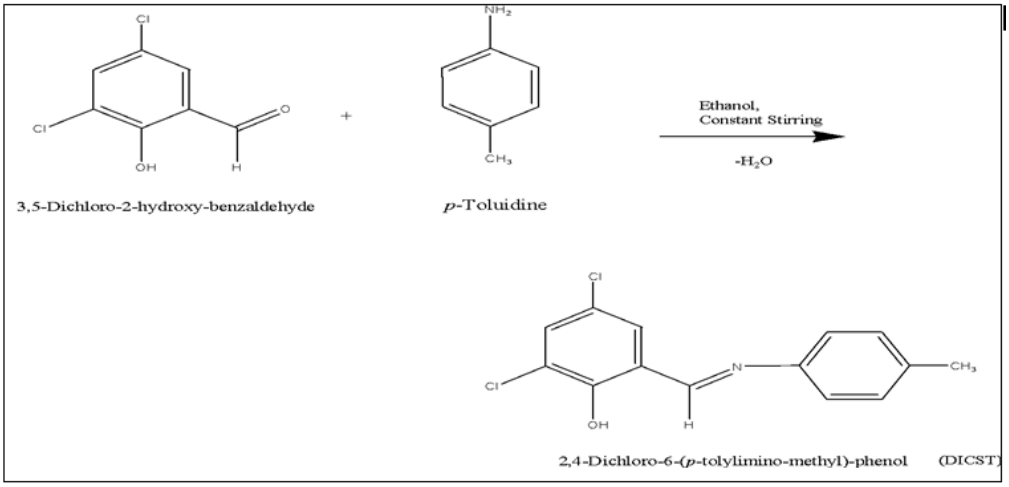
1. INTRODUCTION

Cancer is a major problem both in developed countries and developing countries. Cancer is abnormal; cell growth that can lead to death. Cancer cells are multiplying vigorously and are capable of destroying the normal cells of our body. The cancer cells are produced due to the imbalance in the body and cancer can be treated by correcting this imbalance. Millions of people are affected by cancer every year which may lead to death. According to the World Health Organization, cancer ranks second in global causes of death, with an estimated 9.6 million deaths attributed to this group of diseases in 2018[1-3]. With 36 different types, cancer mainly affects men in the form of colorectal, liver, lung, prostate, and stomach cancer and women in the form of breast, cervix, colorectal, lung, and thyroid cancer[1]. Cancer, characterized by uncontrolled, rapid and pathological proliferation of abnormal cells, is the second[1]leading cause of death in humans after cardiovascular diseases in developing as well as advanced countries.[1] Although there are many therapeutic strategies including chemotherapy and radiotherapy, high toxicity and drug resistance limit the positive outcomes in most cases. Moreover, most chemotherapeutic drugs are expensive. Therefore, novel diagnosis, treatment and prevention approaches are urgently needed for cancer therapy. Research in this direction is still going on, to find an alternative drug that can target the malignant tissues, less toxic and affordable by the common man.[4] Schiff bases played an important role as ligands even a century after their discovery in coordination chemistry. Schiff bases have been widely employed as ligands because of the high stability of the coordination compounds and their good solubility in common solvents. Schiff bases are considered a very important class of organic compounds because of their ability to form complexes with transition metal ions and their pharmacological properties. Transition metal complexes containing Schiff bases have been of much interest over the last years, largely because of their various applications in biological processes and potential applications in designing new therapeutic agents. Recently, Schiff base complexes with transition and inner transition metals[5,6] have acquired special attention in the medicinal and pharmaceutical field, since they exhibit excellent biological activities. Schiff bases are potential anticancer drugs and when administered as their metal complexes, the anticancer activity of the complexes is enhanced compared to the free ligand. In recent times, hydroxy-substituted Schiff bases have received substantial attention due to good anticancer activity. [7,8] Considering the numerous applications of Schiff bases in various fields of chemistry, there has been tremendous interest in evolving proficient methods for their synthesis. Many methods and procedures have been brought in for the preparation of imines in the literature since the pioneering work of Hugo Schiff. Based on the above facts and its applications, the aim of our present research work was designed to evaluate the *in vitro* anticancer activity of the synthesized Schiff base compound 2, 4-dichloro-6-(p-tolylimino-methyl)-phenol (DICST) and its metal complexes of growth inhibitory property using MCF-7, a human breast carcinoma cell line. These synthesized Schiff base ligands and metal complexes have been screened for *in vitro* anticancer activity.

1. MATERIALS AND METHODS

1.1 *Schiff Base (DICST) Synthesis*

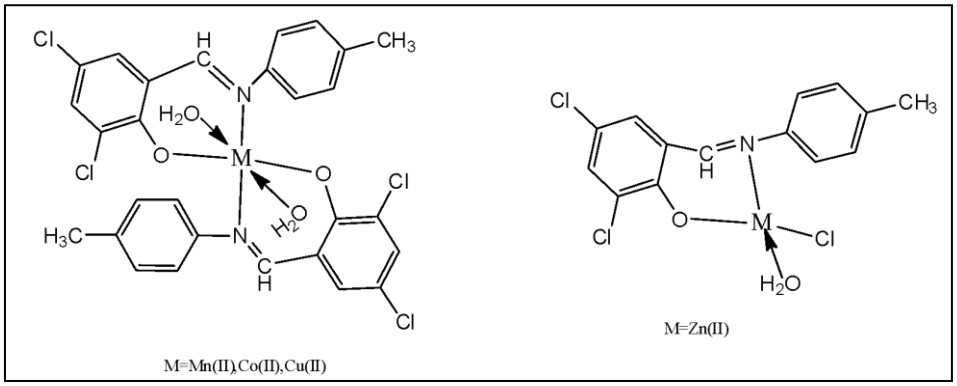
According to the literature method [9,10] the Schiff base of 2, 4-dichloro-6-(p-tolylimino-methyl)-phenol (DICST) was synthesized. To a solution of 3,5-dichloro-2-hydroxy-benzaldehyde (0.05 mol), the solution of p-toluidine (0.05 mol) dissolved in absolute ethanol was added in a 1:1 equimolar ratio and the reaction mixture was stirred for 1-2 hours at 35-40 °C. From absolute ethyl alcohol, the solid product was filtered, washed, and crystallized.



Scheme I. Formation of Schiff base (DICST)

1.2 *Metal Complexes Synthesis*

A warm ethanolic solution of 2,4-dichloro-6-(p-tolylimino-methyl)-phenol (DICST) (0.05 mol) was added to warm ethanolic solution (0.05 mol) of metal salts Mn(II), Co(II), Cu(II) and Zn(II). The resulting mixture was heated in a mantle with a water condenser for 5-6 hours at reflux with continuous shaking. After concentration to one-half of the starting volume, the product was collected as a precipitate, which was cooled, washed with ethanol, and dried in a hot air oven.



Scheme II. Structures of metal complexes

1.3 *In vitro anticancer activity of synthesizing Schiff base transition metal complexes*

1.3.1 *Materials Required*

CO_2 incubator- Sanyo, Japan, Multimode microplate reader- BioTek, USA, Refrigerated centrifuge- Remi, India, Cell: MCF-7 cell line - NCCS Pune, MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) from Sigma, Fetal bovine serum from Genetix Biotech, India. Trypsin from SRL Chemicals, Penicillin/Streptomycin from Sigma, DMEM medium from Genetix Biotech, India and DMSO from SRL chemicals.

1.3.2 *Cell culture*

The breast cancer cell line, MCF-7 was purchased from NCCS Pune and was cultured in liquid medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin.

1.3.3 *MTT assay*

For the MTT assay[11], the cells were grown in 25 cm ×25 cm ×25 cm tissue culture flasks containing DMEM medium as culture medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO) and grown at 37°C under a humidified atmosphere of 95% air and 5% CO_2. Cells were regularly passaged and maintained before including in the experiment. When a cell density in a culture flask reached 70-80% confluence, they were trypsinized and seeded in 96-well plates in the density of 4000 cells per well in 100 µl and incubated for 24 hours at CO_2incubator. The next day, the working stock of 2X concentration (2000, 600, 200, 60, 20 and 6 µg/ml) were prepared in complete DMEM medium supplemented with 10% FCS and were added to the respective wells at 100 µl volume to achieve a final concentration of 1000, 300, 100, 30, 10 and 3µg/ml. After the tested compound addition, the plate was further incubated for 48 hours in the CO_2 incubator at 37°C.MTT solution was composed of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) at 5 mg/ml in 1X PBS. The plate was further incubated for 3 hours in an incubator. After incubation, the medium was carefully decanted from the plate without disturbing the formation of crystals. The crystals were air-dried in dark and dissolved in 100 µl DMSO and the plates were mildly mixed at room temperature for 5 min and the OD was measured using BiotekSynergy HT microplate reader at 570 nm. From the optical densities the percentage growths were calculated using the following formula:

$$\text{Percentage growth} = 100\times[(T-T_0)/(C-T_0)]$$

Where,
T is the optical density of the test,
C is the optical density of control,
T_0 is the optical density at time zero (at the time of compound addition will serve as blank to assess the cytotoxicity).

1.3.4 *Cell Imaging*

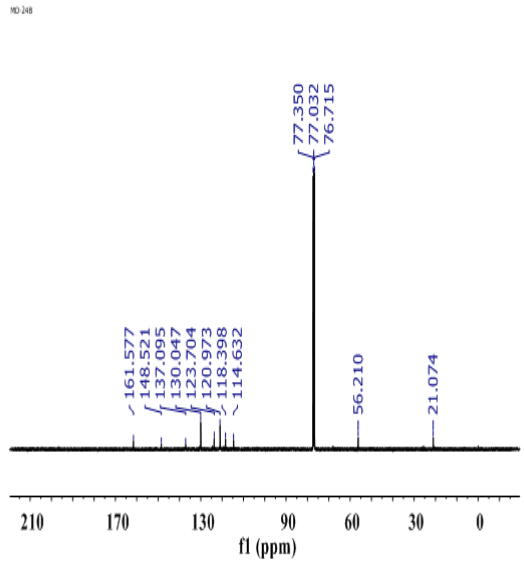
After 48 hours before adding MTT solution, treated cells were observed under a microscope for cell morphology analysis and images of each concentration were captured and recorded.

2. RESULTS AND DISCUSSION

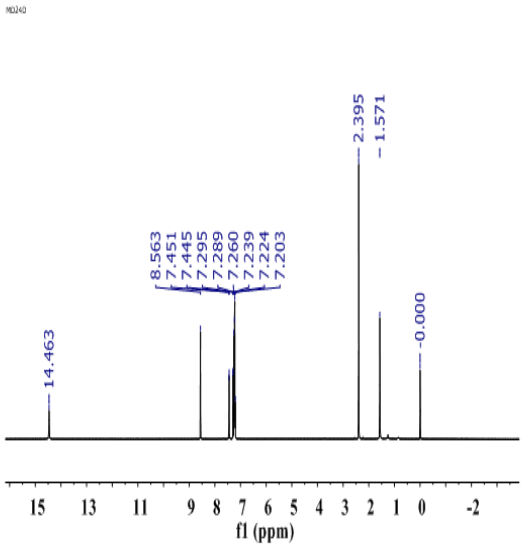
2.1 *NMR spectrum of Schiff base and its transition metal complexes*

2.1.1 *NMR Spectra*

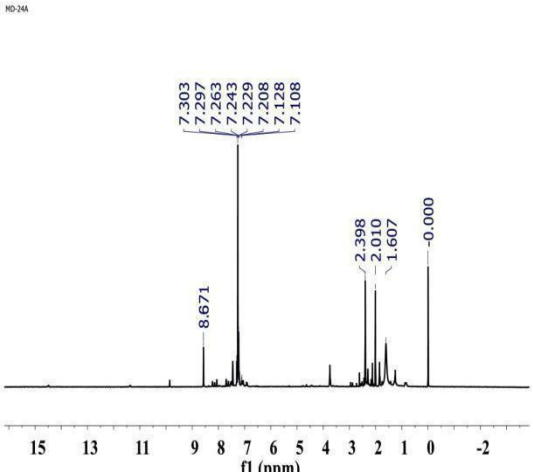
The ^1H and ^{13}C NMR spectra of the 2,4-dichloro-6-(p-tolylimino-methyl)-phenol (DICST) and its Zn(II) complex were studied with DMSO-d_6 as solvent. NMR spectrum were shown in the below Figure-1.



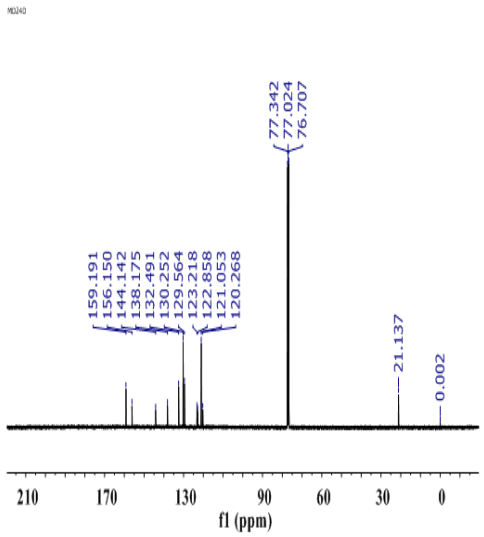
DICST-$ZnCl_2$-C^{13}



DICST-L – H^1



DICST-$ZnCl_2$-H^1



DICST-L-C^{13}

Fig- 1 NMR spectrum of Schiff base and its transition metal complexes

2.2 *^{1}H NMR Spectra*

In Figure-1 ^{1}H NMR spectra of the Schiff base shows that the OH signal appeared in the spectrum of 2,4-dichloro-6-(p-tolylimino-methyl)-phenol (DICST) at 14.46 ppm is completely disappeared in the spectrum of Zn(II) complex indicating that the OH proton is removed by complexation with the metal ion [12]. The singlet peak at 8.56 ppm characteristic to the azomethine[13-15] is down fielded to 8.64 ppm in Zn (II) complex supporting well binding of the azomethine groups of Schiff base to metal ions. As multiples at 7.20-7.40 ppm are down fielded to 7.10-7.30 ppm in the spectra of metal complexes, the signals for aromatic protons of Schiff base were obtained. The previous studies concluded that the ^{1}H NMR spectra of the ligand showed a singlet atδ2.38 ppm attributed to methyl group attached to the thiazole ring, and multiplet at δ7.80–6.93 ppm due to aromatic protons. The spectra showed also azomethine proton (CH=N) as singlet at δ9.19 ppm. The OH proton is observed as singlet at δ11.51 ppm. A comparison of the^{1}H NMR spectra of the Zn (II) complex and the free ligand indicates that the proton signal corresponding to OH group of ligand has disappeared in Zn (II) complex, which may be due to deprotonation. The CH=N proton in the ligand is shifted to 9.08 ppm in the Zn (II) complex, which suggested that the azomethine nitrogen is involved in the co-ordination[16] (Maurya et al., 2005).

2.3 *^{13}C NMR Spectra*

^{13}C NMR spectrum as shown in Figure-1. The signal appeared at 159.19 ppm and was assigned to azomethine carbon atoms (HC=N), in ^{13}C NMR spectra of the 2,4-dichloro-6-(p-tolylimino-methyl)-phenol (DICST). Similarly, with the shift in intensity confirming the coordination of metal ion, the spectra of Zn(II) complex showed a signal of azomethine carbon at 148.52ppm relatively up fielded to the 2,4-dichloro-6-(p-tolylimino-methyl)-phenol (DICST). The phenolic carbon showed the signal at 159.19 ppm (Ph-C-O,

1C) in the ligand and the spectra of the Zn (II) complex showed a signal of phenolic carbon at 161.57 ppm. The signals are shown at 120.2-144.1 and 114.63-148.52 ppm are assigned to aromatic carbons of 2,4-dichloro-6-(p-tolylimino-methyl)-phenol (DICST). [17]Abd-Elzaher *et al*., (2012) reported that the ^{13}C NMR of the ligand showed a signal at 16.3 ppm which was assigned to the methyl group attached to the thiazole rings, and three signals at 120.2, 141.4 and 159.4 ppm were assigned to the thiazole rings.

2.4 *Cell Growth Inhibition Property*

The anti-cancer activity of Schiff base and its transition metal complexes were assessed by the MTT assay method. The tested compounds were tested against the human breast cancer MCF-7 cell line. The percentage of Cytotoxicity of Synthesized Schiff base compound and its transition metal complexes on MCF-7 cell line are shown in Table-1. The concentrations ranging from 1000, 300, 100, 30, 10 and 3µg/ml in the semi-logarithmic range are used to assess the growth inhibition properties of the synthesized compound. These synthesized compounds were screened for their anti-cancer activity against MCF-7 human breast cancer cell lines at different concentrations 3-1000 µg/ml to determine the IC_{50} (50% growth inhibition) by MTT assay was summarized in Table-2. Each concentration was performed in quadruplicate and cumulative variation was maintained less than 20% between the data points. The percentage of cell growth inhibition and cytotoxicity of the synthesized compound on the MCF-7 cell line is shown in Figure-2 and Figure-3. The cell growth inhibition of the synthesized compound revealed that the concentrations increase there is an increase in the cell growth inhibition and possesses anti-cancer activity. The Photomicrograph of the MCF -7 cell line were shown in Figure-4.

Table- 1: percentage of Cytotoxicity of Synthesized Schiff base and its transition metal complexes on MCF-7 cell line by MTT assay

Concentrations (µg/ml)	Tested compound (Schiff base and its transition metal complexes) (%)	Standard (%)
1000	**165.7**	**0.5**
300	**164.8**	**0.2**
100	**146.3**	**2.6**
30	**94.7**	**1.5**
10	**46.9**	**1.8**
3	**-14.5**	**8.6**
Control	**0.0**	**11.3**

At the 0.05 level, the population means are significantly different

Table-2: Percentage of cell growth inhibition of Synthesized Schiff base and its transition metal complexes on MCF- 7 cell line by MTT assay

S. No	Tested sample concentration (µg/ml)	OD Value at 570 nm
1.	**Control**	**0.058**
2.	**1000**	**0.059**
3.	**300**	**0.162**
4.	**100**	**0.474**
5.	**30**	**0.764**
6.	**10**	**1.126**
7.	**3**	**1.121**

At the 0.05 level, the population means are significantly different

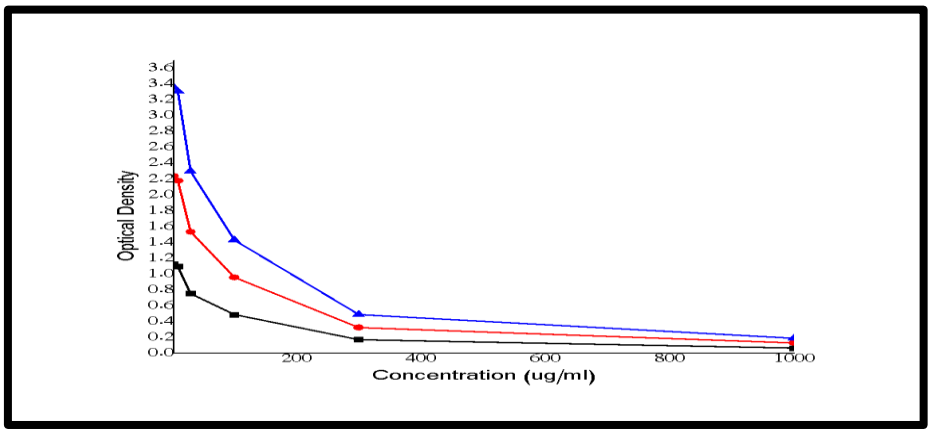


Fig-2: Percentage of cell growth inhibition of synthesized Schiff base and its transition metal complexes on MCF- 7 cell line by MTT assay

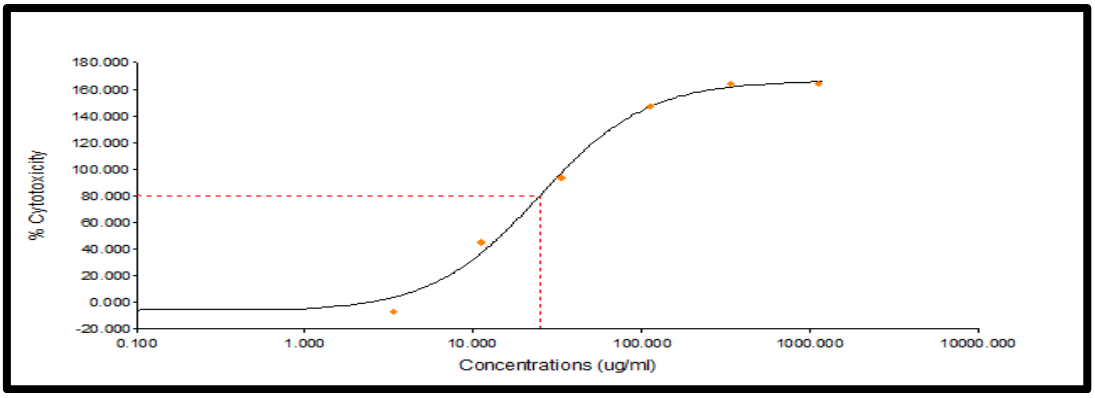


Fig-3: Percentage of cytotoxicity of synthesized Schiff base compound and transition metal complexes on MCF-7 cell line by MTT assay

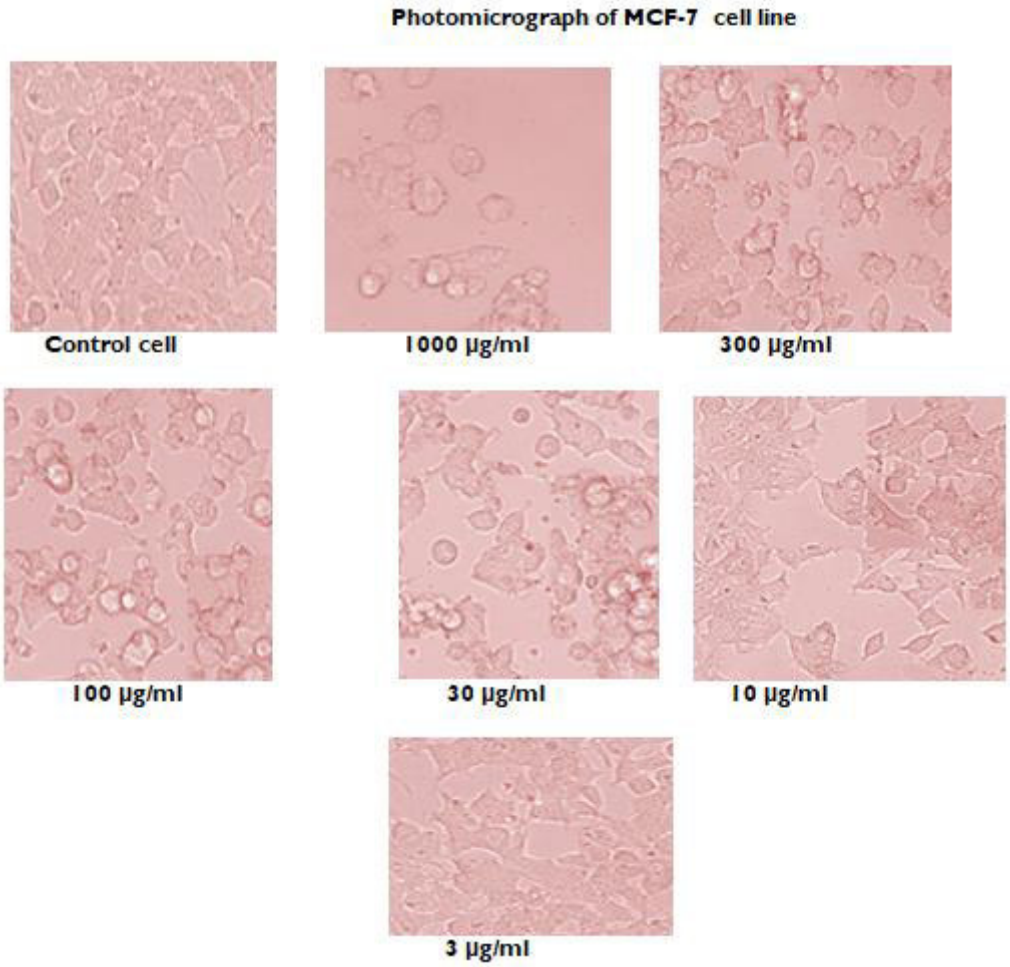


Fig 4: Photomicrograph of MCF -7 cell line (Images of control cells and treated cells)

The tested compounds were screened for their Cytotoxicity against MCF-7 cancer cell lines at different concentrations 1000, 300, 100, 30, 10 and 3µg/ml to determine the IC_{50} (50% growth inhibition) by MTT assay was summarized in the above Tables. The tested compounds showed the cytotoxic effect on the higher concentration tested (Figure 4). The IC_{50} value for the test compounds showed at 24.0 µg/ml on the tested cell line**.** These cell growth inhibitions of the synthesis of Schiff base compound and its transition metal complexes revealed that the concentrations increase there is an increase in the cell growth inhibition and possesses anti-cancer activity. It is concluded that the Schiff base and its metal complexes had anticancer properties against breast carcinoma MCF-7 cell line. A previous study has suggested that the human breast cancer MCF7, cervical cancer cell HeLa K-562 and Vero cell lines at the concentrations of 80, 90, 100, 120, 130 and > 200 mg/mL for 48 h using pyridine Schiff base ligand and its metal complexes. The cell growth inhibition was analysed by the MTT assay (*Shafeeulla et al*) and the results revealed that complexes and the ligand exhibited an inhibitory effect on the proliferation of HeLa, MCF-7 and Vero cell lines in a dose-dependent manner [18].

3. CONCLUSION

Schiff bases are one of the most important chemical classesof compounds having a common integral feature of a variety of medicinal agents. Transition metal complexes containing

Schiff bases have been of much interest over the last years, largely because of their various applications in biological processes and potential applications in designing new therapeutic agents. The results of the present study demonstrate the potential cytotoxic activity of Schiff base and its transition metal complexes that are responsible for this potential cytotoxic activity against the MCF-7 cancer cell line. Further research has to be carried out with other cancer models to elucidate the possible mechanism of action.

4. AUTHORS CONTRIBUTION STATEMENT

K. Sirumalar conceptualized and gathered the data concerning this work Dr.A. Paulraj analyzed these data and necessary inputs were given towards the designing of the manuscript. All authors discussed the methodology, results and equally contributed to the final manuscript.

5. CONFLICT OF INTEREST

Conflict of interest declared none

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