



## Preparation and Characterization of Novel Schiff Base Derived From 4-Nitro Benzaldehyde and Its Cytotoxic Activities

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**Article type:** ABSTRACT

**Original Article**

Normal drugs exhibit activities against both normal and cancer cells. Furthermore, cancer cells may develop resistance to these drugs that alternative treatment must be explored. The main objective of this study was to examine the anticancer activity of Schiff base against Tongue Squamous Cell Carcinoma Fibroblasts (TSCCF) and normal human gingival fibroblasts (NHGF) and to propose its mechanism. A Novel Schiff base ligand was synthesized from the reaction of 5-C-2-4-NABA (5-chloro-2-((4-nitrobenzylidene) amino) benzoic acid). These Schiff bases possessed azomethine group (-HC=N-) and aromatic group (CH) as analyzed by Fourier transforms infrared (FTIR) spectroscopy and UV-Vis spectra. The *in vitro* cytotoxicity screening assay suggested promising activity against TSCCF with IC<sub>50</sub> of 446.68 µg/mL, but insignificant activity against NHGF cells (IC<sub>50</sub> of 977.24 µg/mL) after 72 h. The evidence of apoptotic induction was supported by DAPI staining of apoptotic nuclei with reduced cell numbers, suggesting that Schiff base could induce apoptotic bodies in cancer cells being observed. Based on the Schiff base structure, the anti-cancer mechanism may be attributed to the -HC=N-azomethine group. For the first time, our findings highlighted the anticancer activities of the new Schiff base against oral cancer cell lines.

**Received:**

2023.02.19

**Revised:**

2023.07.28

**Accepted:**

2023.08.05

**Keywords:** Apoptosis, Azomethine group, cytotoxicity, 5-chloro-2-((4-nitrobenzylidene) amino) benzoic acid, oral cancer, Schiff base

**Cite this article:** Abdul Majeed R. Preparation and Characterization of Novel Schiff Base Derived From 4-Nitro Benzaldehyde and Its Cytotoxic Activities. *International Journal of Molecular and Cellular Medicine*. 2022; 11(4):285-296. DOI: 10.22088/IJMCM.BUMS.11.4.285

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Publisher: Babol University of Medical Sciences

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## Introduction

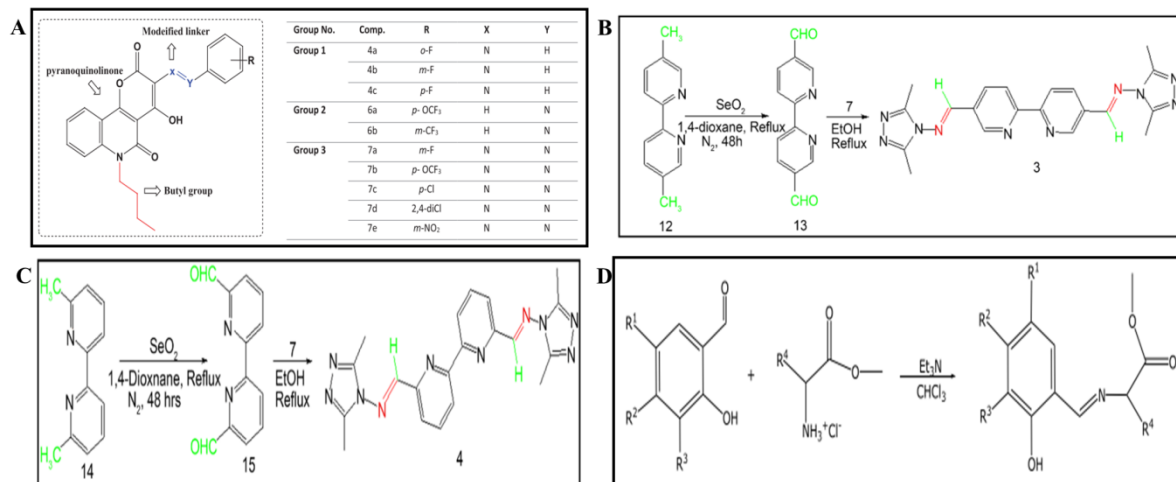
Schiff bases (SBs) have been discovered in 1864 by Hugo Schiff when he studied the condensation of primary amines with carbonyl compounds (1). SBs have been commonly applied as ligands, as ion or molecule that donates a pair of electrons to a metal atom or central ion to form a coordination complex. These are due to their coordination compounds' stability (2) and have played essential role in coordination chemistry development (3). SBs and their complexes have several applications in medicinal inorganic chemists due to their antibacterial, antifungal, antiviral, antimalarial and antineoplastic, antitumor, and anticancer activities and their excellent chelating ability (4,5). Also, some SBs based-pyranoquinolinone show superior inhibitory activity on breast cancer cells (MCF-7) than chemotherapy drugs (Doxorubicin, Dox) (5). Other applications including in organic synthesis, catalysis, liquid crystals, biomimetic modelling applications, and designing molecular magnet particles, and in new technology applications such as liquid crystalline displays, lasers, electro-optical devices, ink-jet printers, and fluorescent properties (6).

There are many common methods that are used to synthesize SBs and their mineral derivatives such as the reaction of aldehydes and ketones with primary amines under acidic or basic conditions, oxidation synthesis of amines from alcohols and amines, condensation of aldehydes, cyanide addition to organo-metallic reagents, the reaction of phenols/phenol ethers with nitriles, the metal amides reaction, preparation of ketamines using ketals, reaction of hydrazoic acid with tertiary alcohols and olefins, transformation of alpha-amino acids to imines, and reduction of nitro-compounds (7). The formation mechanism of the SBs depends on the variety of nucleophiles (amine) addition to the carbonyl group. The amine first reacts with the aldehyde or ketone to yield an unstable compound known as carbinolamine. This compound loses water through acid and /or base-catalyzed pathways. Since carbinolamine is an alcohol, it is subjected to acid-induced dehydration. However, imines hydrolysis is also an essential step in the synthesis of Sonn-muller, Sommelet, Gattermann aldehyde, and Stephen (8).

Cancer is a killer disease, and its therapy is constantly evolving especially for oral cancer. For breast, cervical, and colon cancers, the drugs normally used are 5-fluorouracil (5-FU), tamoxifen, and bevacizumab. These however have become less effective, as cancer cells may develop resistance to these drugs (4). The challenge is to develop new drugs to control drug resistance and their side effects. The mechanisms of the anticancer activity of different compounds as novel, effective, and safe anticancer drugs have been reviewed. However, converting mineral complexes into medicines fit for human consumption is challenging, due to the aggregation of metal ions in the body fluids which can be hazardous. Thus, the bio-compatibility of minerals and their bio-efficiency is a necessary factor to be taken first into account (4,9). Tongue squamous cell carcinoma (TSCC) is the most common oral cancer cell and is known for its high proliferation rate and nodal metastasis (10), which is mediated by various proteolytic enzymes and angiogenesis (11). Oral cancer development may result from common pathways involving untreated inflammation and high levels of cytokines pro-inflammatory (12).

Schiff bases and azo dyes prepared from 3-amino-4-hydroxy-2H-pyrano[3,2-c]quinoline-2,5(6H)-dione (Figure 1A), have shown cytotoxic activities against MCF-7, HePG2, and HCT-116 cells (5). A new Schiff base synthesized from 1,10-phenanthroline-2,9-dicarboxaldehyde and from 2,2'-bipyridyl-4,4-dialdehyde (Figure 1B and C) (13), exhibits significant and selective anticancer activities against BT549 and A549 cells.

Schiff base (Figure 1D) also prepared from amino acid methyl esters with salicylaldehyde derivatives (2,4-dihydroxybenzaldehyde, 2-hydroxy-3-methoxybenzaldehyde, and 5-bromo-2-hydroxybenzaldehyde) and have shown strong activity against different cancer cells (14). In this study, preparation and characterizations of novel SBs derived from 4-nitro benzaldehyde were carried out, and the anticancer activity of SB against Tongue Squamous Cell Carcinoma Fibroblasts (TSCCF) and normal human gingival fibroblast (NHGF) was investigated. To our knowledge, there has yet to be any study examining the effects of SBs on TSCC and NGF.



**Fig.1.** Design strategies of the Schiff bases that have been prepared from different compounds: (A) synthesis from 3-amino-4-hydroxy-2H-pyrano[3,2-c]quinoline-2,5(6H)-dione; (B) 1,10-phenanthroline-2,9-dicarboxaldehyde; (C) from 2,2'-bipyridyl-4,4'-dialdehyde; (D) synthesis from amino acid methyl esters with salicylaldehyde derivatives (2,4-dihydroxybenzaldehyde, 2-hydroxy-3-methoxybenzaldehyde, and 5-bromo-2-hydroxybenzaldehyde).

## Materials and methods

All chemicals and reagents (5-C-2-4-NABA (5-chloro-2-((4-nitrobenzylidene) amino) benzoic acid), absolute ethanol) utilized in this study were laboratory standard and purchased from Aldrich and Prolabo BDH (VWR). The IR spectra were recorded in the range 400-4000  $\text{cm}^{-1}$  using FTIR 8400 S-Shimadzu Spectro-photometer, at room temperature. The melting points of ligands were determined by the electro-thermal fisher device while the electronic spectra were detected using UV-1800 Shimadzu.

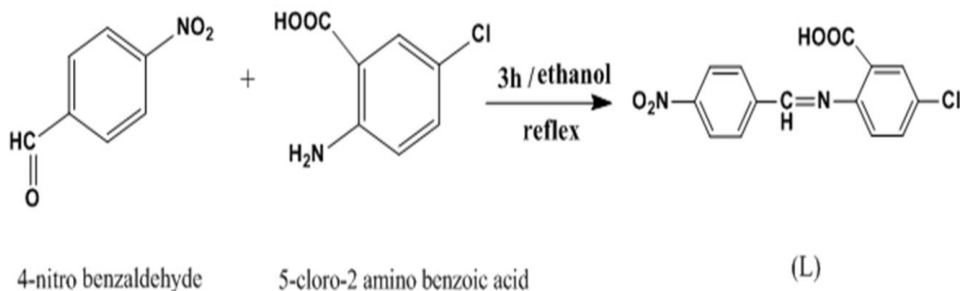
### Synthesis of SB ligand

The SB ligand is schematically shown in Figure 2, synthesized by the reaction of hot absolute ethanol (40 mL) of 4-nitro benzaldehyde (1 gm, 10 mmol), and hot absolute ethanol (30 mL) of 5-chloro-2 amino benzoic acid (10 mmol) with a few drops of glacial acetic acid. This mixture was condensed for 3 h at room temperature at melting point 182-185  $^{\circ}\text{C}$  (Figure 2). The mixture sediment was filtered and dried to remove ethanol.

### Characterization of SB ligand

#### Fourier transforms infrared (FTIR) spectroscopy

The pellets of the prepared SB were re-dispersed 3 times in sterile distilled water (DW) to eliminate any contaminated biological particles and to ensure removal of free components from the SB ligand.



**Fig.2.** Synthesis of 5-C-2-4-NABA (5-chloro-2-((4-nitrobenzylidene)amino)benzoic acid)

The pellets were purified, dried, and the powder was mixed with potassium bromide (KBr) and subjected to FTIR spectroscopy measurement (FTIR –84005-Shimadzu, Japan) in the diffuse reflectance mode at a resolution of 400-4000  $\text{cm}^{-1}$  at room temperature in the laboratories of Department of Chemistry, College of Education and Pure Sciences, University of Basrah.

#### The UV–Vis spectra and Nuclear magnetic resonance spectra (NMR)

The UV–Vis spectra were measured with Shimadzu UV-1800 spectrophotometer at Marine Sciences Center, University of Basrah. A concentration of  $1 \times 10^{-3}$  M in dimethylformamide solvent (DMF) was prepared for the measurements. The  $^1\text{H-NMR}$  spectra (Oxford, US) was measured as previously reported by Saeed *et al.* (5).

#### Cytotoxic activity

##### Cell culture

The NHGF cell line was obtained from the Pasteur Institute (Tehran, Iran) and the TSCCF cell line was obtained from the Iranian Biological Resource Center (Tehran, Iran). Cells were cultured in alpha-minimum essential medium ( $\alpha$ -MEM, Life Technologies, Gibco, Waltham, MA, USA) supplemented with 10 % FBS (fetal bovine serum, BioWest S.A.S., Nuaille, France), 20  $\mu\text{g/mL}$  penicillin (100 U), and 100  $\mu\text{g/mL}$  streptomycin (Sigma-Aldrich®, St. Louis, MO, USA) in an incubator at 37 °C and 5 %  $\text{CO}_2$ . After reaching confluency of about 75 %, cells were detached using 0.25 % trypsin (Invitrogen, Gibco, Waltham, MA, USA) and ethylenediaminetetraacetic acid (0.1 %, Merck, Darmstadt, Germany) in PBS (phosphate-buffered saline) at 37°C. Cells were then re-suspended in  $\alpha$ -MEM with 1 % PSF and 10 % FBS.

##### MTT Assay for cytotoxic activity

Cells were cultured in the 96 well plates at  $5 \times 10^3$  cells per well and incubated for 24 h to grow. The cells then were washed with PBS (pH 7.4) and incubated for 72 h in  $\alpha$ -MEM media containing different concentrations of SB (1000, 500, 250, 125, 62.5, 0  $\mu\text{g/mL}$ ). The viability of the cell was measured using the 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich®, USA) assay. After incubation for 72 h, MTT (0.5  $\text{mg/mL}$  in PBS) was loaded to each well, and incubated for 4 h at 37 °C. The formazan was dissolved by adding 100  $\mu\text{L}$  of DMSO to each well with gentle shaking at 37 °C, and the

absorbance was measured using an ELISA reader (Bio-Rad Laboratories) at 570 nm. Concentrations of SB showing a 50 % reduction in oral cell viability ( $IC_{50}$  values) were then determined using Graph Pad Prism (Version 6).

#### DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride) staining

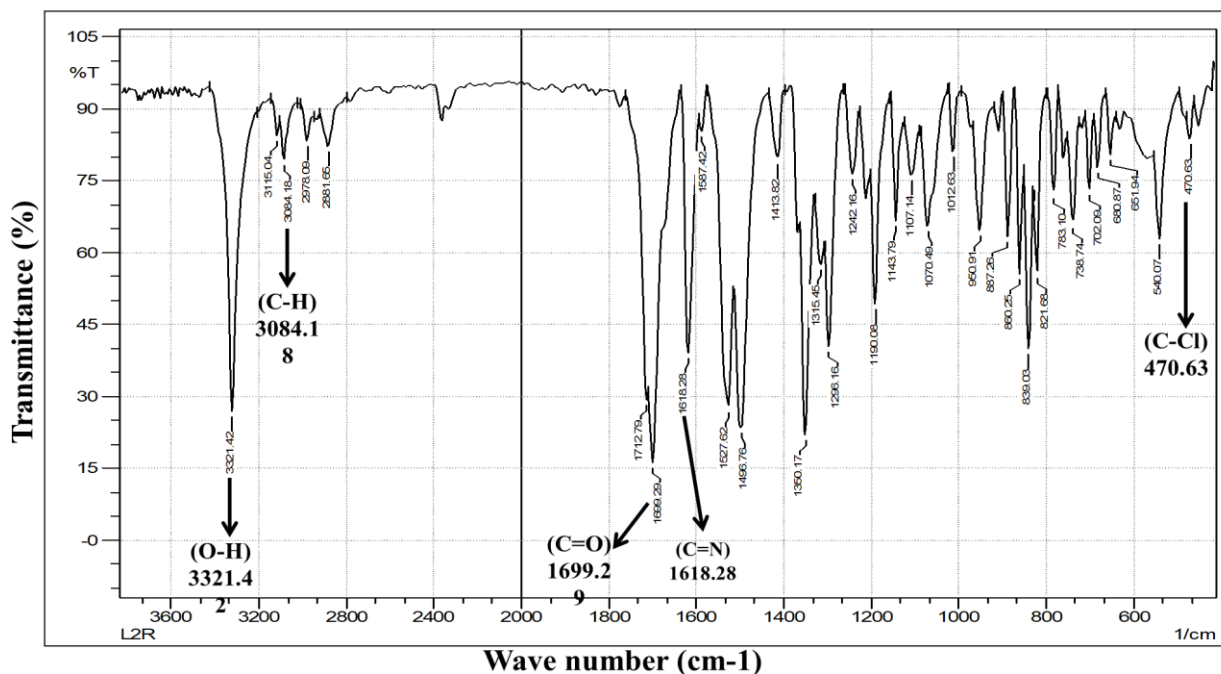
After 72 h of culture, cells were fixed with 4 % glutaraldehyde solution (Sigma-Aldrich, USA) and stained for 20 min with DAPI (Sigma-Aldrich, USA) in a dark condition. The apoptotic bodies were detected using a fluorescent microscope.

#### Statistical Analysis

The data were introduced as mean  $\pm$  SEM. One-way ANOVA was used for statistical analysis using Graph Pad Prism (Version 6). The results were measured to be statistically significant at  $p < 0.05$ .

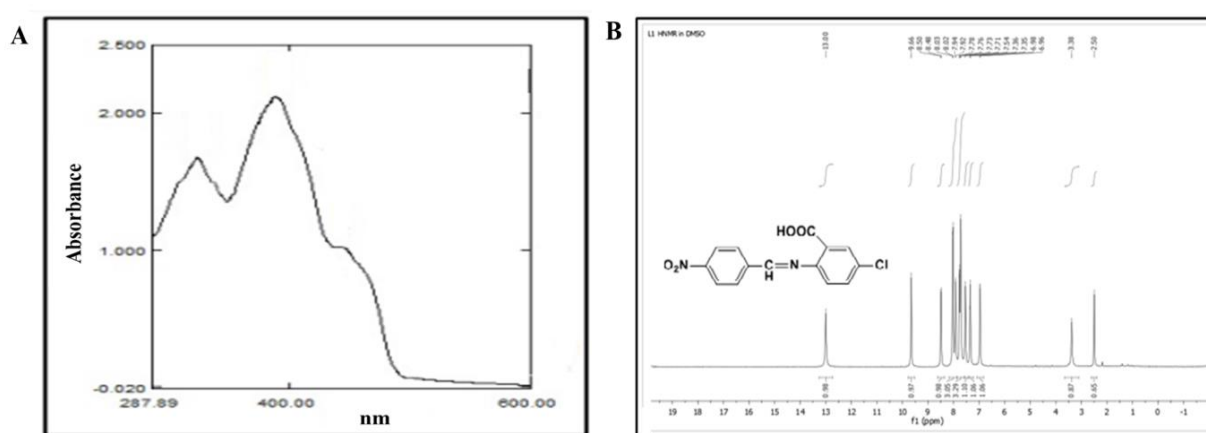
### Results

In the present study, 4-nitro benzaldehyde was used to prepare the SB ligand and the proposed structure was presented in Figure 2, based on IR and UV/ visible spectroscopic analyses. The band of IR spectra at  $1618.28\text{ cm}^{-1}$  was attributed to  $\text{-HC=N-}$  (azomethine group), which are condensation compounds of ketones or aldehydes with primary amines (8). The band at  $3321.42\text{ cm}^{-1}$  was due to (COOH) group and the weak absorption band at  $3084.18\text{ cm}^{-1}$  was assigned to the (CH) aromatic group. There was also a band at  $1699.29$  belonging to the (C=O) group and also a weak band at  $470.83$  belonging to the (C-Cl) group (Figure 3).



**Fig.3.** IR Spectrum of the Schiff base ligand.

The visible and ultraviolet spectrum of the ligand (L) (Figure 4A) showed three absorption peaks. The first peak at 291 nm was due to the transition  $\pi-\pi^*$  of the double bond in the aromatic rings, the second one at 400 nm was due to the transition  $\pi-\pi^*$  in the azomethine group, and the third peak at 460 nm may be attributed to the transition  $\pi-\pi^*$  resulting from the total internal succession in the ligand. The  $n-\pi^*$  peak may be hidden under the third peak. The  $^1\text{H-NMR}$  spectrum of the ligand (L1) as shown in Figure 4B showed the following signals: a signal at  $\delta$  9.66 ppm (s) belongs to the proton of the azomethine group ( $-\text{HC}=\text{N}-$ ) and the signals between  $\delta$  6.99 and 8.50 ppm belong to the aromatic protons. The signal at  $\delta$  13 ppm (s) refers to the (OH) group of the phenyl in the benzoic acid, while the signal at  $\delta$  2.5 ppm (s) refers to the protons of DMSO, and the signal at  $\delta$  3.38 ppm (s) refers to the water in DMSO.



**Fig.4.** Ultraviolet-visible spectra (a) and  $^1\text{H-NMR}$  spectra (b) of the Schiff base ligand.

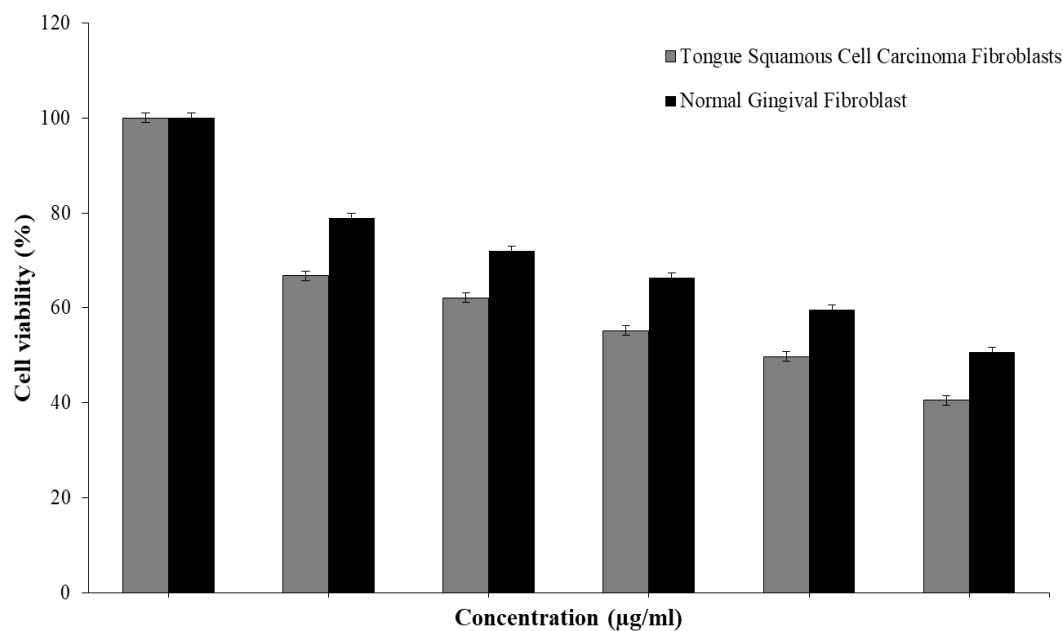
### Cytotoxic activity

Chemotherapy drugs must have the ability to destroy cancer cells without affecting normal healthy cells. Novel anticancer agents should be able to control multiple tumorigenic occurrences and their mechanisms, with stronger selectivity towards cancer cells and with lower or non-toxicity against normal cells (4,15). The *in-vitro* antitumor activity of SB at different concentrations was evaluated in NHGF and TSCCF cell lines. There was a reduction in cell viability of TSCCF cancer cells after 72 h treated with SB at  $\text{IC}_{50}$  of 446.68  $\mu\text{g/mL}$ , while a slight decrease of NHGF cell viability was observed after 72 h treatment with  $\text{IC}_{50}$  of 977.24  $\mu\text{g/mL}$  as shown in Figure 5 and Table 1. These results indicate that the SB exhibited cytotoxic activity against oral cancer TSCCF cell lines, but with less toxic effects against NHGF.

**Table 1.** Anticancer activity ( $\text{IC}_{50}$ ) of (5-chloro-2-((4-nitrobenzylidene)amino)benzoic acid) (or 5-C-2-4-NABA) against TSFC and NGF cells after 72 h treatment.

Cell line	$\text{IC}_{50}$ ( $\mu\text{g/mL}$ )
Tongue squamous cell carcinoma	446.68
Normal gingival fibroblast	977.24





**Fig.5.** The IC<sub>50</sub> value of Schiff base on NHGF and TSCCF cell-lines after 72 h treatments.

### DAPI staining

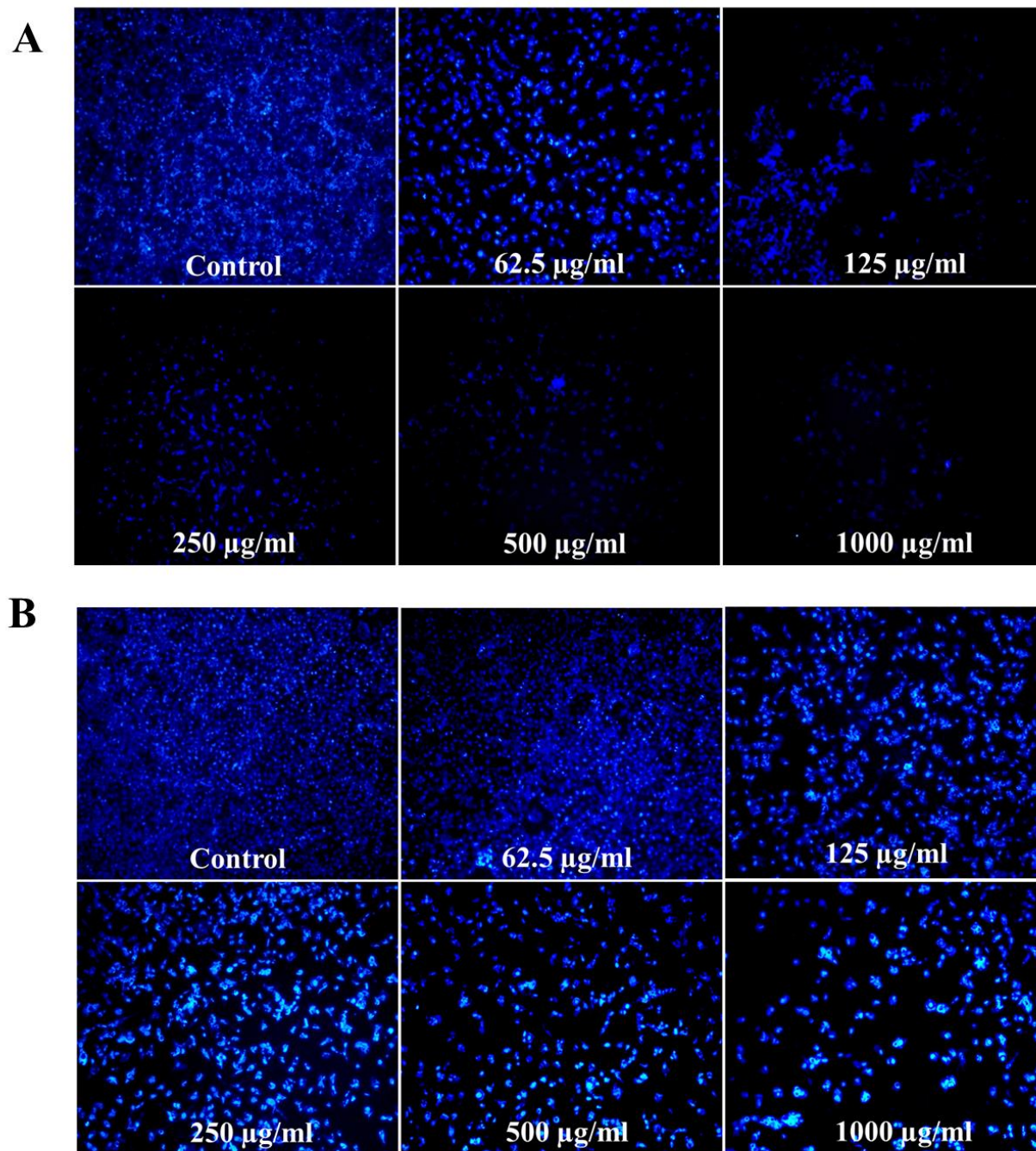
The nuclear fragmentation induced by SB was observed by DAPI staining, a common nuclear counterstain, using a fluorescence microscope. DAPI can bind to adenine-thymine-rich sequences of the DNA minor groove to form a fluorescent complex while a non-fluorescent compound is formed through the insertion (16). The percentage of SB-treated cells stained by DAPI was significantly different from the untreated cells. For TSCCF, the control cells showed normal nuclei (smooth nuclear) whereas the treated cells showed apoptotic nuclei (fragmented or condensed chromatin) and reduced cell numbers. There was an increased number of apoptotic cells, especially at higher concentrations, suggesting that SB could induce apoptotic bodies in a cancer cells as shown in Figure 6A. Insignificant morphological changes were observed with NHGF cells as shown in Figure 6B and Table 2.

**Table 2.** Anticancer activity of the reported SBs against different cell-lines.

Schiff Base type	Cell lines	IC <sub>50</sub>	Duration Time (h)	Ref.
Schiff base 2,2'-((1E,1'E)-((2,2-dimethylpropane 1,3-diyl)bis(azanylylidene))bis(methanylylidene))bis(4-fluorophenol) (L2F) and Pd (II) complex (PdL2F)	human colorectal carcinoma (HCT116)	90.00 and 4.10 µg/mL		(24)
Schiff Bases: Synthesized from 4-Amino -3,5-dimethyl-1, 2, 4-triazole, Phenathroline and Bipyridine Dicarboxaldehydes	human lung carcinoma (A549), breast carcinoma (BT549), prostate adenocarcinoma (PC3) and mouse preadipocytes (3T3-L1) cells	70, 50, and 1/4200 µM		(13)

Schiff bases and azo dyes derived from 3-amino-4-hydroxy-2H-pyrano[3,2-c]quinoline-2,5(6H)-dione	HepG-2, HCT-116, and MCF-7	1.82, 6.49, and 8.06 $\mu\text{g/mL}$		(5)
Schiff base ligand, 2-((E)-((4-((E)-benzylidene)amino) phenyl) imino)methyl) -naphthalene -1-ol-Cu(II) complex	PC-3, SKOV3, and HeLa	0.161, 0.063, and 0.087 $\mu\text{g/mL}$	72 h	(25)
Schiff base: salicylaldehyde with 2-amino-4-phenyl-5-methyl thiazole.	MCF-7, HepG2, A549 and HCT116	6.20 to 9.22 and from 6.00 to 10.00 $\mu\text{g mL}^{-1}$	48 h	(26)
Schiff base ligands, R-(phenyl)methanamine (L1), R-(pyridin-2-yl)methanamine (L2), and R-(furan-2-yl)methanamine (L3) (R-(E)-N-((1H-pyrrol-2-yl)methylene))	Caco-2, HeLa, HepG2, MCF-7, and PC-3) and noncancerous (MCF-12A)	>100	24 h	(27)
Morpholine schiff base ligand	MCF7	100 $\mu\text{M}$	48 h	(28)
Water-soluble Schiff Base containing S, O, N heteroatoms by the condensation reaction from 4-amino-3-hydroxynaphthalene-1-sulfonic acid and 2-hydroxy-3-methoxybenzaldehyde and its Cu (II), Zn (II) and Ni (II) complexes	A549 cells	Ligand (L) and nickel (II) complex (L-Ni) did not able to inhibit A549 cell proliferation while L-Cu and L-Zn had $\text{IC}_{50}$ at 12 and 80 $\mu\text{M}$	24 h	(29)
Schiff base ligand derived from 4-chloro-o-phenylenediamine and 3,5-dichloro-2-hydroxyacetophenone	MCF-7	2.5 – 100 $\mu\text{g/mL}$	36 h	(30)
Schiff base is 4-(((10-chloroanthracene-9-yl)methylene)amino)-1,5-dimethyl 1,2-phenyl-1,2-dihydro-3H-pyrazole-3-one	MCF-7	< 0.1 mM	72 h	(31)
Schiff base is 4-(((8-hydroquinoline-2-yl)methylene)amino)-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazole-3-one	MCF-7	0.14 mM	72 h	(31)
Schiff bases derived from aminobenzothiazole derivatives with Salicylaldehyde/Bromosalicylaldehyde	MCF-7	44.12 $\mu\text{M}$	72 h	(32)
Schiff bases containing benzothiazole unit	MCF-7	200 $\mu\text{g/mL}$	72 h	(33)
Bis-Schiff bases of pyrazoles 9–24	HepG2 MCF-7 RPE-1 (normal retina pigmented epithelium)	84.2 $\mu\text{M}$ 99.4 $\mu\text{M}$ 127.7 $\mu\text{M}$	48 h	(34)
4,5,6,7-tetrahydrobenzo[d]thiazole-based Schiff-bases	HepG2 MCF-7	1.29 $\mu\text{M}$ 34.52 $\mu\text{M}$	48 h	(17)
Schiff base derived from 4,4'-[1,4-phenylenebis(1,3,4-thiadiazole-5,2-diyl)] bis (azaneylylidene) bis (methaneylylidene) diphenol	MCF-7	250 $\mu\text{g/mL}$	72 h	(35)
Tetronic Schiff bases	A549 cell line	39.63 $\mu\text{M}$	72 h	(36)





**Fig.6.** The DAPI nuclear staining of NHGF (a) and TSCCF (b) control cells and Schiff base treated cells exhibited a condensed form of nuclear materials in apoptotic cells.

## Discussion

The -HC=N- group is considered the main structural component for the biological activity of SB (17). SB ligands are distinctive because they can be easily synthesized by condensing a single pot of primary amines and aldehydes. These compounds and metal complexes are advantageous for clinical, analytical, industrial, and catalytic applications (18). The electronic spectra of the ligand were recorded in DMF between 250-600 nm. UV-VIS is a very advantageous analytical device used to study the spectral properties, sensitivity, substitution effect, pH, and ambient temperature of SB. Generally, the absorbance of SB was detected between 300 to 450 nm using UV-Vis spectrometry. However, this is highly dependent on the solvent, the identical Z or E isomer, the solvent-based electron density transfer, and the substitution position (7).

The lipidic nature of the cell membrane makes it extremely permeable to lipophilic molecules and increases the delocalization of lipophilicity and  $\pi$ -electrons. There is a direct correlation between lipid solubility, bioactivity, and bioavailability as lipophilicity could reduce solubility and cell dissociation barriers leading to enhanced bioavailability and bioactivity of the molecule (19).

The known organic compounds show high biological activities, especially those that contain heteroatoms such as sulfur, oxygen, and nitrogen in addition to the aromatic ring, or those with multiple bonds in their structure and a high electron density. A high inhibition efficiency can be due to strong coordination bond, and also the presence of hetero-atoms in the order  $O < N < S < P$  which promote the inhibitory activities of carcinogenic compounds (20). The higher suppression efficiency of the SB than the aldehydes and amines can be correlated to the -C=N- group in the molecules (21). The potent anticancer activity of 2-Acetylpyridine N-substituted thiosemicarbazone ligands and its complexes on MCF-7 breast cancer cells is due to the terminal phenyl group in their structures (14). Our results confirm that SB contains effective functional groups, including chloride, carboxyl groups, nitrogen, and a benzene ring, with electronic densities that confer significant biological effects on oral cancer cells.

The anticancer activity of SB can be attributed to the presence of a (-COOH) group in the site close to the phenyl ring; the presence of a greater ratio of Chlorine in the SB; and the presence of an N-butyl group and the alkyl chain length. The cytotoxic activities against cancer cells will increase with the increase in the length of the N-alkyl chain and this has been suggested due to the improvement in lipophilicity leading to enhanced cell membrane penetration of the SB (5). The -NO<sub>2</sub> and -OH groups of the benzene rings and the entire structure of the compounds at different doses may prove to be safer treatment in the future against different cancer types (22). Table 2 summarizes the anticancer activity of the reported SBs against different cell lines. DAPI analysis showed significant apoptotic changes in cancer cells such as nuclear fragmentation, condensation of chromatin, and formation of apoptotic bodies (23). DAPI is only slightly permeable into living cells but is highly permeable in dead cells (16). Our study provided insights into the potential use of SB as a safe and cost-effective oral cancer treatment. Further studies are needed to explain the specific mechanism leading to the inhibition of cancer cell growth as well as validation in *in vivo* animal model before its future development as new therapeutic drugs.

In this study, a novel SB ligand was synthesized and characterized using UV-Vis, FTIR and <sup>1</sup>H-NMR. The synthesized SB exhibited cytotoxic activity against oral cancer TSCCF cell lines, but with less toxic

effects against NHGF cells. A DAPI staining analysis suggested that the new SB may have induced cytotoxicity through apoptotic pathways, as the treated cells showed nuclear fragmentation and membrane blebbing. The anticancer activity of SB was suggested due to the presence of a (-COOH) group in the site close to the phenyl ring; and the presence of a greater ratio of Chlorine, and an N-butyl group and the alkyl chain length.

### Acknowledgements

The authors thank the University of Basra for the research opportunities.

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