SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL ACTIVITY OF4-CHLORO-2-{[(*E*)-PYRIDIN-2-YLMETHYLIDENE]AMINO}BENZOIC ACID WITH COBALT(II) COMPLEX

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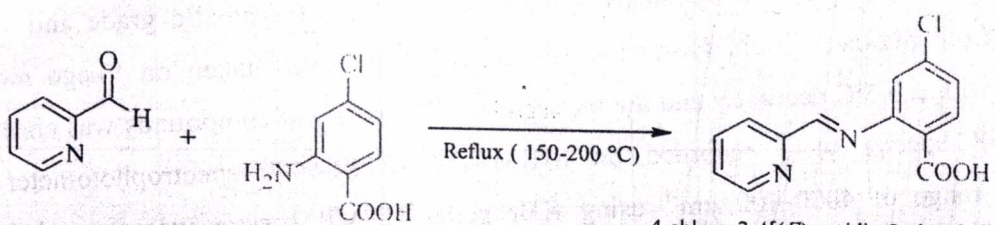
ABSTRACT:

Synthesis of*4-chloro-2-{[(E)-pyridin-2-ylmethylidene]amino}benzoic acid (SB-1)*from the reaction of2-pyridine carboxyaldehydeand 2-amino-4-chlorobenzoic acid in ethanol. The Schiff base is reacted with cobalt chloride in acetonitrile and solution of two equivalent of triphenylphospine to form the corresponding *4-chloro-2-{[(E)-pyridin-2-ylmethylidene]amino}benzoic acid cobalt complex.*It is characterized by chemical properties and spectroscopic data. These compounds were tested for anticancer,anti-inflammatory activity and antimicrobial activity against a variety of test organisms: *Escherichia coli, Staphylococcus aureus, Candidaalbicans.* Especiallychlorogroup as substituent on the phenyl ringis shown to contribute substantially to the antimicrobial activity.

Graphical Abstract



4-chloro-2-{[(*E*)-pyridin-2-ylmethylidene] amino}benzoic acid (SB-1)

Keywords: Schiff base, Cobalt complex, anticancer, anti-inflammatory, antimicrobial activity.

1. INTRODUCTION:

The field of Schiff base complexes is fast developing because of the wide variety of possible structures for the ligands. Schiff base are organic compounds possessing azomethine group which resulted from condensation of amine with aldehyde or ketone. Schiff base ligands are essential in the field of coordination chemistry, especially in the development of complexes of Schiff bases because these compounds are potentially capable of forming stable complexes with metal ions [1]Such type of ligands represents vast utilized classes of new series of compounds in coordination chemistry [2]. Schiff bases are organic compounds with great utility in various fields [3] such as medicine, agriculture, cosmetic products etc. Recently, Schiff base complexes have drawn attention in biochemistry and biomedicine because of their unique properties [4,5]. Schiff bases are important precursors forthe synthesis of some bioactive compounds [6,7]. Schiff bases have received considerable attention since the discovery of their antibacterial [8,9], antifungal [10], anti-HIV [11,12], anti-inflammatory [13], anticonvulsant[14,15], antiviral [16], antimalarial, anti-proliferative, and antipyretic activities [17-18] and anticancer properties [19]. The presence of the inimical grouping in these organic ligands plays an important part in manifesting these biological characteristics[20].

The aim of the resent study was to prepare, characterize and determine the anticancer, antiinflametry, antimicrobial properties of 4-chloro-2-{[(E)-pyridin-2-ylmethylidene]amino}benzoic acid ligand and their cobalt metal complex for pharmaceutical uses.

2. MATERIAL AND METHODS

All chemicals used in synthesis of compounds were of synthetic grade and were procured from Sigma-Aldrich, Hi-media. All melting points were taken on Veego model VMP-DS with ± 0.5°C accuracy and are uncorrected. The purity of compounds was checked by TLC.IR spectra were recorded on SHIMADZU-FTIR-8400 spectrophotometer in frequency range of 4000-400 cm^{-1} using KBr pallet.^{1}HNMR spectra were recorded on BRUKER spectrometer (400 MHz) using $CDCl_3$ as a solvent and TMS as an internal reference.

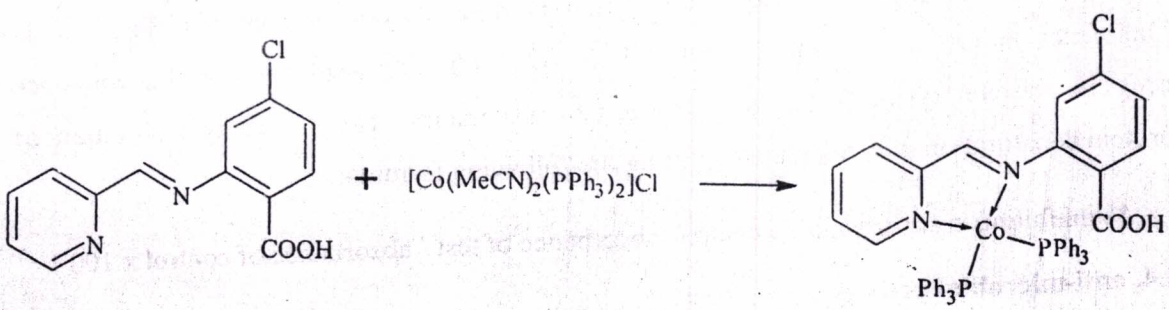
2.1.Synthesis of 4-chloro-2-{[(E)-pyridin-2-ylmethylidene]amino}benzoic acid (SB-1):

A reaction mixture of 2-pyridine carboxyaldenyde(0.01mol) and 2-amino 4-chlorobenzoic acid(0.01mol), and ethanol (10ml) was refluxed at 150-200°C in oil bath for 3-4 hrs, reaction was monitored through TLC. Recrystallized in ethanol to obtained compound **(SB-1)**[21].

It is white crystalline solid; yield-4.70g; m. p. 88°C;
IR spectrum (KBr pellets), υ (cm^{-1}): 1651.63 (C=N str.), 1495.03 (C=C str.), 1304.07 (C-N str.) and 737.92 (C-Cl).^{1}HNMR spectrum (δ ppm): 7.208-7.389 (4H, m, pyridine-H); 7.809-8.181 (3H, m, Cl-Ar-H) 8.576 (1H, s, H) and 10.1 (1H,s, Acidic H).
^{13}CNMR spectrum (δ ppm): 122.03, 122.46, 129.06 (Cl-Ar-CH), 125.38, 136.82 (Cl-Ar-C), 149.21-149.33, 154.23 (Pyridine CH), 154.19 (HC=N), 160.89(COOH).

2.2.Synthesis Cobalt of 4-chloro-2-{[(E)-pyridin-2-ylmethylidene]amino}benzoic acid complex (CoSB-1):

To a solution of cobalt chloride (1mol) in a 10 ml acetonitrile ,a solution of two equivalent of triphenylphospine was added. The reaction mixture was stirred for 30 min at room temperature and allowed to evaporate slowly. The crystalline product obtained was subsequently added to a stirred solution of 4-chloro-*N*-[(*E*)-pyridin-2-ylmethylide-ne]anilineligand(1 mol) in 10 ml dichloromethane for 2 hrs and solution was evaporated to small volume under vacuum. The yellow coloured complex were developed by diffusion of diethyl ether into the solution



It is yellow crystalline solid; yield-1.9g; m. p. 88°C.IR spectrum (KBr pellets), υ (cm^{-1}): 1651.63 (C=N str.), 1495.03 (C=C str.), 1304.07 (C-N str.) and 737.92 (C-Cl). ^{1}HNMR spectrum (δ ppm): 7.210-7.347 (2H, m, Cl-Ar-H); 7.785-8.184 (4H, m, pyridine-H) and 8.577 (1H, s, H). C^{13}NMR spectrum (δ ppm): 122.01-122.46,129.08-129.35 (Cl-Ar-CH), 136 (Cl-Ar-CH), 149.37, 149.74, 154.23 (Pyridine CH), 160.91 (HC=N).

2.3. In Vitro Antitumor Activity

2.3.1. Cell Culture.

Thecellswere routinely cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum. Theculture was maintained at 37◦C with a gas mixture of 5%CO_2/95% air. The medium was changed every two days andthe cells were sub cultured every three days.

2.3.2. Cell Viability Assay.

Cell viability was determinedusing the MTT assay. Briefly, the cells were collected andresuspended in RPMI1640 medium at $4^{\times}10^4$ cells/mL,100 μL aliquots were added to each well of 96-well flatbottomedmicrotiter plates, followed by addition of 100 μLof the SB1 and complexe.Three replicate wells were used foreach data point in the experiments. After incubation for theindicated intervals, 20 μL ofMTT(5mg/mL in PBS) solutionwas added to each well and plates were then incubated for4h at 37◦C. The medium with MTT was removed fromthe wells. Intracellular formazan crystals were dissolved byadding 150 μL of DMSO to each well, and the plates wereshaken for 10min.The absorbance was read at 550 nm witha microplate reader. Percentage of survival was calculated asa fraction of the negative control (medium only). The halfmaximalinhibitory concentration (IC50) was obtained.

2.3.In vitro anti-inflammatory activity

The reaction mixture (10 mL) consisted of 0.4 mL of egg albumin (from fresh hen's egg), 5.6 mL of phosphate buffered saline (PBS, pH 6.4) and 4 mL of synthetic derivatives (1000, 800, 600. 400, 200µg/ml). Similar volume of double-distilled water served as control. Then the mixtures were incubated at (37^0c ±2) in a incubator for 15 min and then heated at 70°c for 5 min. After cooling, their absorbance was measured at 660 nm by using vehicle as blank. Diclofenac sodium at concentration 1000, 800, 600. 400, 200µg/ml) was used as reference drug and treated similarly for determination of absorbance. The percentage inhibition of protein denaturation was calculated by using the following formula,

% inhibition = absorbance of control -absorbance of test / absorbance of control x 100

2.4. anti-microbial activity

SB1 and complex were screened for in vitro antibacterial activity against three pathogens viz. Gram positive bacteria (*Staphylococcusaureus*(ATCC no.6538); Gram-negative bacteria (*Escherichia coli* (ATCC no. 8739) *and yeast Candida albicans (*ATCC no. 10231*)* by taking DMSO as a negative control. Agar well diffusion method was performed to calculate the zone of inhibition where bacterial strains were subcultured in the nutrient broth

and concentration of compounds 100 µL were prepared [22]. Bacterial strains were spread on the agar plate with the help of spreader and 8 mm diameter wells were dug with the help of sterile metallic borer. Above concentration of compounds were introduced into the well with the help of sterilised tips of micropipette and incubated it at 37 °C for 24-48 h for bacte'ia and 20-25°C for yeast. The zones of inhibition were noted and compared with the standard drug.

3. Results and discussion:

3.1.Spectral Discussion:

The IR spectrum of compound SB-1 showed a strong carbonyl stretching absorption band was observed at 1746cm^{-1}. A characteristic band of C=C stretching observed at 1495.03 cm^{-1}. The stretching at 1651.63 cm^{-1} indicated the presence of C=N that means 2-pyridine carboxyaldehyde was joined to 2-amino-4-chlorobenzoic acid *via* amine group. The C-N stretching was observed at 1304.07 cm^{-1} and C-Cl stretching found at 737.92 cm^{-1}.

The ^{1}HNMR spectrum in $CDCl_3$ showed 4 shifts for 9 protons which was same in number with molecular formula. The multiplets peak at δ 7.208-7.389 due to four protons of pyridine ring. The multiplet peaks observed at δ 7.809-8.181 for three H of 2-amino-4-chlorobenzoic acid ring. The characteristic singlet peak of acidic proton was seen at δ 10.1 and singlet peak was observed at 8.577 for single proton showed the attachment of aldehyde with 2-amino-4-chlorobenzoic acid.^{13}CNMR spectrum showed the peak at 122.03-122.46, 129.06, 125.38, 136.82 for aromatic carbons of 2-amino-4-chlorobenzoic acidring and for pyridine ring at 149.21-149.33, 154.23. The peak for carbon to which carboxylic group was attached showed δ value at 160.89 and for carbon of C=N group showed peak at 154.19.

3.2 Anti-Cancer activity:

Chemotherapy is the major approach for both localized andmetastasized cancer. Therefore, the synthesized compoundswere screened for their *in vitro* cytotoxicity and growth inhibitoryactivities against human tumor cell linesie. liver cancer cell line HepG2. The screening results are given in Table below.

SB1 and Complex were evaluated for their ability to inhibit the growth of HepG2 human hepatoma cell lines usingMTT assay. The inhibition was expressed as cell viability relative to control. In the presentstudy, HepG2 human hepatoma cellswere used which have been recently characterized as asuitable model for *in vitro* assessment of hepatoma toxicity[23, 24].

And flurouracil(5-FU, 30 μM) was used asa positive control, which has been used extensively as anefficient anticancer drug in clinical trials.

Anti-Cancer:$[Co(SBL_1)(PPh_3)_2Cl_2$								
Sr. no.	**Sample**	**ABS T1**	**ABS T2**	**ABS T3**	**Mean O.D.**	**% of cell viability**	**% of cell inhibition**	**IC 50**
1	**Control**	0.312	0.311	0.313	0.312			--

As shown in Table the oxovanadium SB1 and complex exhibit broad inhibition on the human cancer cell lines with the IC50 values ranging from1.68to 55.40 μM, respectively.The results indicate that both exhibit antiproliferativeeffect to human hepatoma cells HepG2 in a time and does-dependent manner with increasingthe concentrations of SB1 and complex.The IC50 values of complexHepG2 cells after treated for24h,less than that of complex. It suggests that SB1 possessed more potentinhibitory effect against the cancer cells.This difference maybe attributed to the introduction of chlorine.

Anti-Cancer: 4-chloro-2-{[(E)-pyridin-2-ylmethylidene]amino}benzoic acid [Schiff Base]								
Sr.No	**SB-1**	**ABS T1**	**ABS T2**	**ABS T3**	**Mean O.D**	**%of Cell Viability**	**%of Cell Inhibition on**	**IC.50**
1.	**Control**	0.312	0.311	0.313	0.312	--------	--------	--------
2.	**200 µg/ml**	0.222	0.213	0.201	0.212	67.95	32.05	620.92
3.	**400µg/ml**	0.196	0.195	0.191	0.194	62.18	37.82	
4.	**600 µg/ml**	0.181	0.182	0.179	0.180	57.70	42.30	
5.	**800 µg/ml**	0.131	0.134	0.131	0.132	42.31	57.69	
6.	**1000µg/ml**	0.074	0.070	0.072	0.072	23.08	76.92	

2	200 µg/ml	0.186	0.188	0.181	0.185	59.30	40.70	877.85
3	400 µg/ml	0.179	0.181	0.174	0.178	57.06	42.94	
4	600 µg/ml	0.163	0.169	0.163	0.165	52.89	47.11	
5	800 µg/ml	0.160	0.152	0.159	0.157	50.33	49.67	
6	1000 µg/ml	0.145	0.153	0.152	0.150	48.08	51.92	

3.2.Anti-Inflammatory:

Inflammation is a body response to injury which is characterized by redness, pain ,heatand disturbed physiological function. Inflammation is a protective responseto tissue injury caused by physical, chemical trauma or any microbial agents. It isresponse of the body to inactivate the invading organism to remove the irritant andto allow the body for repair of tissues[25]. Inflammatory inhibition of synthesizedcompounds and reference drug sodium diclofenac was calculated for percentageinhibition of protein denaturation in fresh egg albumin. Values and presented in Table.

Concentration (µg/ ml)	Diclofenac Sodium(Abs)	% inhibition
200	0.13	88.07
400	0.11	89.90
600	0.07	93.57
800	0.05	95.41
1000	0.04	96.33

4-chloro-2-{[(E)-pyridin-2-ylmethylidene]amino}benzoic acid [Schiff Base]

Concentration (µg/ ml)	SB-1	% inhibition
200	0.34	68.80
400	0.20	81.65
600	0.07	93.57
800	--	--
1000	--	--

$[Co(SBL_1)(PPh_3)_2Cl_2]$

Concentration (µg/ ml)	$[Co(SB\text{-}1)(pph_3)Cl_2]$	% inhibition
200	0.70	35.77
400	0.25	77.06
600	0.10	**90.82**
800	--	--
1000	--	--

1. The SB1 and complex exhibit a varying degree of the percentage inhibitionfrom 10.34 to 18.68% at 200 µg/mLto 1000µg/mLconcentrations and order of inhibitionvalue.
2. Anti inflammatory activity is dependent more or less on the concentration ofcompounds. As the concentration increases there is increase in the inhibitionpercentage of denaturation .
3. Compounds SB1have % inhibition value equal to the standard drug(93.57) at 600 µg/ ml concentration and the complex have % inhibition value very near to the standard drug (90.82) at 600 µg/ ml. Both compounds was most potent anti-inflammatorycompounds and might be beneficial for the treatment of inflammationrelated diseases.
4. The exact mechanism of action was not known but according to the proposedmechanism these compounds inhibit the protein denaturation which results in theinhibition of water retention and adema formation. Thus the inhibition of ademaformation leads to the inhibition of inflammation[28].

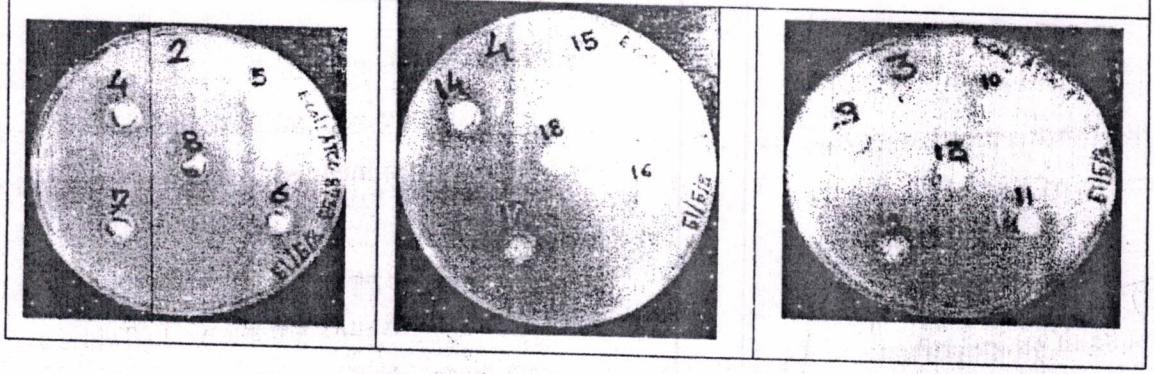
*3.3. Anti- Microbial*activity:

The antimicrobial activity is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by known concentrations of the isolated substance to be examined against a reference substance.During the study it has been found that some drug isolates inhibiting the growth of test organisms because of its antimicrobial property. Schiff base and complex was weak antimicrobial against E. Coli. Based on the results following is conclusion

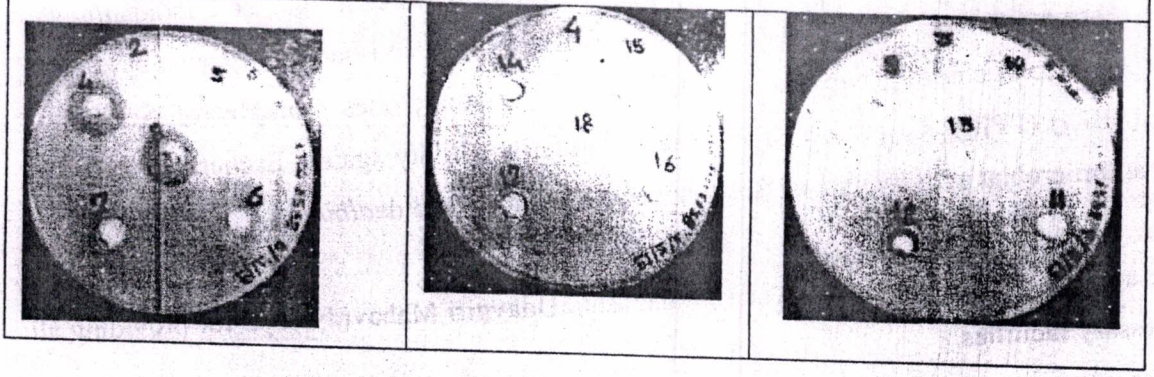
Plate ID	Sample ID	E. coli (Zone in mm)	S.aureus (Zone in mm)	Candida albicans (Zone in mm)
17	*Standard*	23.42 Antimicrobial	32.17 Antimicrobial	13.16 No Antimicrobial
7	*(Schiff Base)*	13.01 Weak Antimicrobial	12.21 No Antimicrobial	13.83 No Antimicrobial
11	Complex	14.70 Weak Antimicrobial	13.00 No Antimicrobial	14.66 Weak Antimicrobial

Weak significant – zone above 12 mm and below 14, Significant antimicrobial- zone above 14 mm based on diameter of agar cup and diluents interference

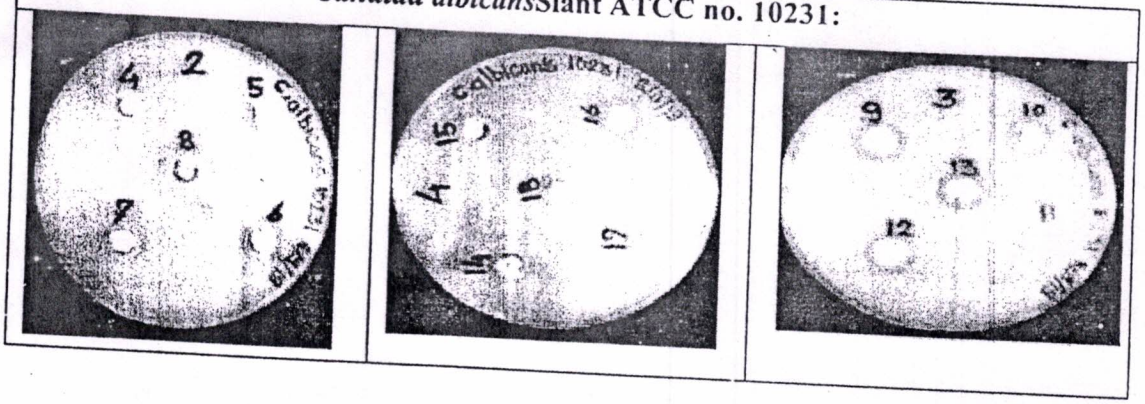
Echerchia coli ATCC no. 8739.



Staphylococcus aureus Slant ATCC no.6538 ;



*Candida albicans*Slant ATCC no. 10231:



4. CONCLUSION:

The Schiff-base ligand **1** (*4-chloro-2-{[(E)-pyridin-2-ylmethylidene]amino}benzoic acid (SB-1)* and *Cobalt 4-chloro-2-{[(E)-pyridin-2-ylmethylidene]amino}benzoic acid complex* was synthesized and determined with different spectroscopic techniques.

The new compounds were investigated for in vitro cytotoxicity for four human tumor cell lines. Schiff bases possess a high potential toinhibit carcinoma cells which enhanced with complexation but the mechanism of their anticancer activity is not confirmed.From results of anti-inflammatory studies it was observed that all synthetic compound exerts steady and significant antiinflammatory actions. This results is also recommended that antiinflammatory actions of synthetic compounds is due to attached groups.

The results of the antimicrobial screening of the Schiff bases against all bacteria have been found. The inhibition zones were measured in mm and results are shown in Table. The results of antimicrobial screening, indicate that Schiff bases show significant activity against Staphylococcus aureus, Escherichia coli, Candida*albicans*.Schiff base 4-Chloro-N-[(E)-pyridin-2-ylmethylidene]anilinewere found to be weak significant against Candida*albicans* and more active against Staphylococcus aureus, Escherichia coli bacterial strains because of the presence of chloro group which itself is active against microbes. Complex of Schiff base **[$Co(SBL_1)$ $(PPh_3)_2$ Cl_2]** show SignificantAntibacterial activity against Escherichia coli, and No antimicrobial activity against Staphylococcus aureus, Candida*albicans*.

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