Synthesis, Spectroscopic Characterization and Biological Activity of Pyridine Schiff Base Ligands by MTT Assay

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Abstract:

Herein we report the synthesis of a series of 4-chloro-N-{[(E)-pyridine-2-yl methylidene]aniline,4-chloro-2-{[E)-pyridin-2-yl methylidene]amino}benzoic acid,4-chloro-2-{[(1E)-1-(pyridin-2-yl) ethylidene]amino}benzoic acid by reflux 2-pyridine carboxyaldehyde,4-chloroaniline,2 amino -4-chlorobenzoic acid,2- acetyl pyridine in ethanol at 150-200^{0}C for 3-4 Hrs. The synthesized compounds were characterized with CHN analysis, IR , ^{1}HNMR ,^{13}C NMR. Synthesized compounds were screened for their In-vitro cytotoxicity and growth inhibitory activities against Human Tumor cell lines .i.e. Liver Cancer cell line HepG$_2$.Synthesized compounds were tested for anti-microbial, anti-cancer and anti-inflammatory activities against Staphylococcus aurous, Candida albicans, Escherichia coli. we observed that the ligand SB2 (4-chloro-2-{[E)-pyridin-2-yl methylidene] amino benzoic acid) possessed more potent inhibitory effect against the Cancer Cells. This is due to introduction of chlorine group in the main moiety.

Keywords : Azomethine group, Human Hepatoma Cell lines (HepG2), Liver cancer cell line, Anti-proliferative effect, Percentage of inhibition, Inflammation, Gram- positive, Gram- negative bacteria ,Yeast.

1 INTRODUCTION

The field of Schiff base metal complexes is fast developing due to wide variety of possible structures for the ligands. Schiff bases are organic compounds having Azomethine group which resulted from condensation of amine with aldehyde or ketone. Schiff base ligands are important in the field of Co-ordination Chemistry forming stable

complexes with metal ions.[1]Now a days, Schiff bases have drawn attention in bio-chemistry and bio- medicine due to their unique characterization and properties.[2-3]These observations paved new avenues to synthesize some 4-chloro-{[(E)-pyridin-2-yl-methylidene]}aniline. Schiff bases with view to investigate their strength as excellent chemotherapeutic agent.

Recent years they are having considerable attention due to its use in different areas of biological activities. The Schiff bases are known to exhibit anti-microbial[4], anti-inflammatory[5],anti-cancer[6]properties.Schiff bases have received considerable attention since the discovery of their anti-bacterial[7,8],anti-fungal[9],anti- HIV[10,11],anti-viral activities.[12]

In this paper ,we report that synthesis of a series of 4-chloro-N-{[(E)-pyridin-2-yl methylidene]}anine,4- chloro-2-{[(E)-pyridin-2-yl methylidene]amino}benzoic acid,4-chloro-2-{[(1E) –1-(pyridin-2-yl)ethylidene]amino }benzoic acid by continuous condensation reactions of 2-pyridine carboxyaldehyde and 4-chloroaniline (1 mole), 2-pyridene carboxyaldehyde(1 mole) and 2–amino 4-chloro benzoic acid(1 mole), 2 –Acetyl pyridine (1 mole) and 2-amino-4-chloro benzoic acid (1 mole)and ethanol (10 Ml) was refluxed at 150-200^{o}C in 100 mL round bottom flask (R.B.F) with 2-3 pieces of porcelain ,continuous condensation for 3-4 hours. The synthesized compounds were confirmed and characterized by CHN analysis,IR,^{1}HNMR and their biological (anti-microbial and anti- cancer) activities.

2 MATERIALS AND METHODS

All chemicals and reagent used in the synthesis of compounds were of synthetic grade and procured from Sigma –Aldrich, Hi-Media company.[15,17]IR spectra were recorded on SHIMADZU –FTIR -8400 Spectrophotometer in frequency range 4000-400 cm^{-1} using KBr pellets.^{1}HNMR spectra were recorded on BRUKER Spectrometer (400MHz) using $CDCl_3$ as a solvent and TMS as an internal reference.

3 SYNTHESIS OF SCHIFF BASES

Schiff bases were prepared by refluxing a mixture of 2- pyridine carboxyaldehyde (1mole) and 4-chloro aniline (1mole) and ethanol (10ml) was refluxed at150-200 ^{O}C in

oil bath for 3-4 hours and cooled. After cooling, the colored solid products were filtered and washed with cold ethanol and vacuum dried over $CaCl_2$.The purity of the products was checked through TLC(Thin Layer Chromatography).

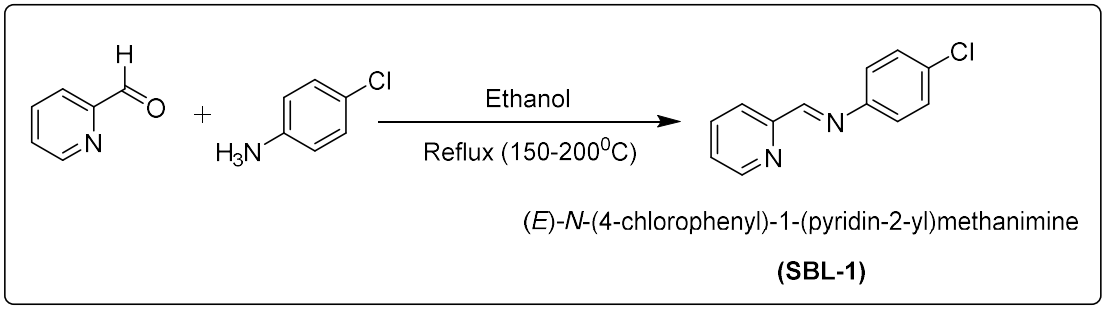
Name of Synthesized Schiff bases and their representation are as follows:-

HL-1 1 4-chloro-N- {[(E)-pyridin-2-yl methylidene]}aniline.

HL-2 2 4-chloro-2-{[(E)-pyridin-2-yl methylidene] amino }benzoic acid.

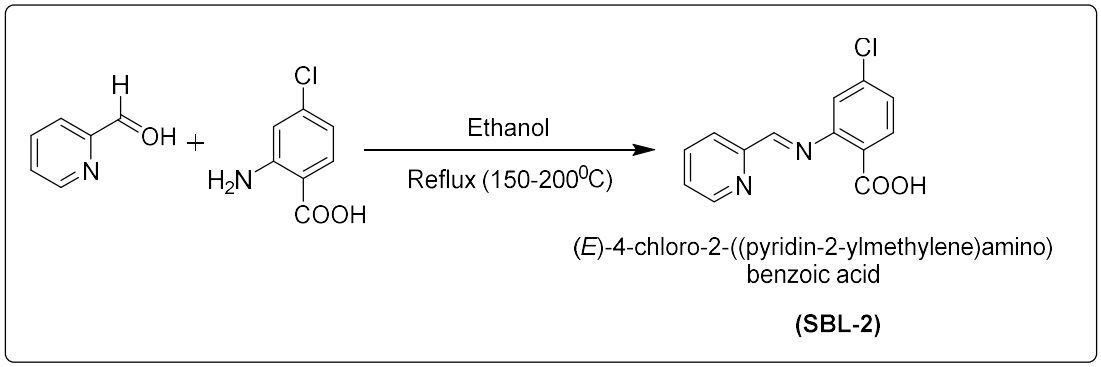
HL-3 3 4-chloro-2- {[(1E)-1-(pyridine-2-yl)ethylidene]amino }benzoic acid.

Schemes:-



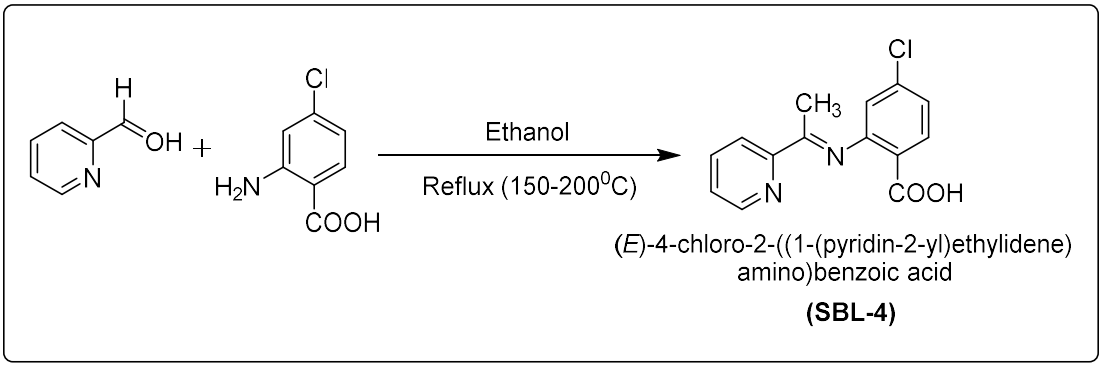
(*E*)-*N*-(4-chlorophenyl)-1-(pyridin-2-yl)methanimine

(SBL-1)



(*E*)-4-chloro-2-((pyridin-2-ylmethylene)amino) benzoic acid

(SBL-2)



(*E*)-4-chloro-2-((1-(pyridin-2-yl)ethylidene) amino)benzoic acid

(SBL-4)

4. RESULTS AND DISCUSSION

Elemental and Physical Constant data of Schiff bases:

Synthesized Schiff bases with theoretical and experimental values considering elemental analysis of compounds confirmed through Stoichiometry.

^{1}H-NMR Spectroscopic Studies:

The ^{1}HNMR spectrum in $CDCl_3$ showed proton signal at ɗ 7.210-7.347 ppm is observed due to two protons of 4- chloro aniline ring .Another multiplets peak observed at ɗ 7.785-8.184 for four H of 2-pyridene carboxyaldehyde ring. An additional singlet peak was observed at ɗ 8.577 for single proton showed the attachment of aldehyde with chloroaniline group. For the ligand HL-2 the multiplets peak at ɗ7.208-7.389 due to four protons of pyridine ring. The multiplet peaks observed at ɗ 7.809-8.181. For three H of 2- amino -4-chlorobenzoic acid ring. The characteristic singlet peak of acidic proton was seen at ɗ 10.1 & singlet peak was observed at ɗ 8.577 for the single proton showed the attachment of aldehyde with 2-amino-4-chlorobenzoic acid. For HL-3 showed 4 shifts for11 protons which was same in number with molecular formula.

IR SPECTROSCOPIC STUDIES:

IR spectra provided important information about the possible co-ordination structures. Valuable absorption bands of ligands are confirmed by IR spectrum. Both signals due to $\sqrt{}_{C=C}$ &$\sqrt{}_{C=N}$ at 1495.03,1651.63 cm^{-1}respectively.The C-N stretching was observed at 1304.07 cm^{-1} and C-Cl stretching found at 737.92 cm^{-1}.

Electronic Spectral Analysis:

Electronic spectra of all ligands are characterized by two bands in Ultra –Violet visible region. The occurring bands in the range 252-290 nm are due to low or medium energy $\pi \rightarrow \pi *$ transition within aromatic ring moieties.Due to perturbed local excitation of the phenyl group such type of transitions may originate.Band in lower energy region of the spectra between 335-350 nm is attributed to the n$\rightarrow \pi^{*}$ transition of azomethine group .Electronic spectral data of compound are as shown in table.

^{13}C-NMR Spectroscopic Studies:

^{13}C-NMR spectrum showed the peak at 122.01-122.46,129.08-129.35 for aromatic carbons of Chloroaniline ring and for pyridine ring at 149.37,149.74,154.23. The peak for carbon to which chloro group was attached shows d̀ value at 136 ppm for HL-1 (d̀ppm)122.01, 122.46,129.08-129.35 (Cl-Ar-CH)136 (Cl-Ar-CH),149.37,149.74,154.239 (pyridine CH),160.91 (HC=N),For HL-2 the peak at 122.03-122.46,129.06,125.38,136.82 for aromatic carbons of 2- amino 4-chlorobenzoic acid ring and for pyridine ring at 149.21, 149.33,154.23.The peak for carbon to which carboxylic group was attached showed d̀ value at 160.89 and for carbon of C=N group showed peak at 154.19.

Table – 1:Elemental and Physical constant data of Schiff Bases.

Sr. No	Name of Schiff Bases	Mol.Formula	M.P(^{0}C)	Mol. Weight	Color	Elemental Analysis		
						C% Found(Cal.)65.87	H % Found(Calc.) 5.10	N % Found(Calc.) 13.01
1	HL-1	$C_{12}H_{10}N_2cl$	261^0 C	217.5	Pale Brown	66.20	4.60	12.87
2	HL-2	$C_{13}H_9N_2O_2Cl$	312^0 C	260.5	White	59.88	3.45	10.74
3	HL-3	$C_{14}H_{11}N_2O_2Cl$	318^0 C	274.5	White Yellow	61.20	4.00	10.20

Table – 2:NMR data of Schiff Bases.

Sr. No.	**Schiff Bases**	**NMR Signals in (d̀ppm)**
1	HL-1	7.210-7.347(2H,m,Cl-Ar-H),7.785-8.184(4H,m,pyridine-H)and

		8.577(1H,S,H)
2	HL-2	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,AcidicH)
3	HL-3	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,AcidicH)

Table – 3: IR Spectral data of the Schiff bases (cm^{-1})

Sr.No.	Name of Schiff Bases	$\sqrt{}_{C=N}$	$\sqrt{}_{C=C}$	$\sqrt{}_{C-N}$	$\sqrt{}_{C-Cl}$	$\sqrt{}_{C-COOH}$
1	HL-1	1651.63	1495.03	1304.07	737.92	-
2	HL-2	1661.06	1538.58	1348.52	738.03	2921.49
3	HL-3	1659.82	1506.04	1307.04	738.17	2924.08

Table 4:- UV-Visible λmax values in nm of Schiff bases.

Sr. No.	Schiff Bases	$\pi \rightarrow \pi^*$	$n \rightarrow \pi^*$
1	HL-1	290	340
2	HL-2	252	350
3	HL-3	260	335

Table 5: ^{13}C-NMR data of Schiff bases.

Sr. No	Schiff Bases	NMR Signals in (dppm)
1	HL-1	122.01-122.46,129.08-129.35(Cl-Ar-CH),136(Cl-Ar-c),149.37,149.74,154.23(pyridine CH),160.91(HC=N)
2	HL-2	122.03, 122.46,129.06(Cl-Ar-CH),125.38(Cl-Ar-CH),136.82(Cl-Ar-C),149.21, 149.33,154.23(pyridine CH),154.19(HC=N),160.89(COOH)
3	HL-3	122.03, 122.46,129,129.06(Cl-Ar-CH),125.38(Cl-Ar-CH),136.82(Cl-Ar-C),149.21, 149.33,154.23(pyridine CH),154.19(HC=N),160.89(COOH)

Table – 6: Anti-microbial Activity of Schiff Bases

Sr. No.	Schiff Bases	*Escherchia coli*	*Staphylococcus aureus*	*Candida albicans*
1	HL-1	+++	+++	+
2	HL-2	++	+	+++
3	HL-3	+++	+++	+++

(Weak Significant Zone above 12mm and below 14(+), significant anti-microbial-Zone above 14mm (Highly active +++), moderately active (++)).

Table – 7: Anti-microbial Activity of Schiff Bases

Plate ID	Sample ID	*Escherchia coli (Zone in mm)*	*Staphylococcus aureus (Zone in mm)*	*Candida albicans (Zone in mm)*
5	HL-1	14.33	14.90	12.53
7	HL-2	13.01	12.31	13.83
6	HL-3	14.99	14.52	14.63
19	DMSO	12.66	14.10	12.40
20	Standard	23.42	32.17	13.16

(Weak Significant Zone above 12mm and below 14, significant anti-microbial-Zone above 14mm based on diameter of agar Cup and diluents interference).

Table – 8: Anti-Cancer studies of Schiff Base on MTT assay using (liver cancer cell line Hep G2)

8.1 Effect of HL-1 against Hep G2 cell line (liver cancer cell line) by MTT assay.

Sr.No.	HL-1	ABS T_1	ABS T_2	ABS T_3	Mean O.D	%of cell viability	% of cell inhibition	Ic_{50}
1	Control	0.312	0.311	0.313	0.312			390.83
2	200µg/ml	0.000	0.000	0.000	0.000	0.000		
3	400µg/ml	0.143	0.144	0.145	0.144	46.16	53.84	
4	600µg/ml	0.128	0.128	0.131	0.129	41.35	58.65	
5	800µg/ml	0.109	0.110	0.108	0.109	34.94	65.06	
6	1000µg/ml	0.048	0.046	0.053	0.049	15.71	84.29	

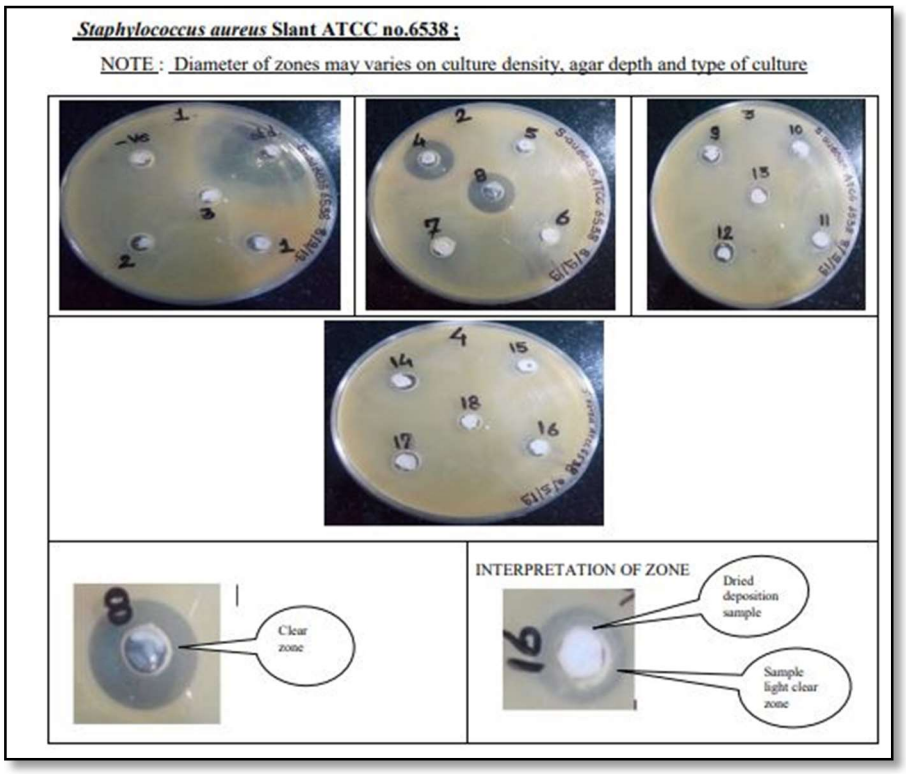
8.2 Effect of HL-2 against Hep G2 cell line (liver cancer cell line) by MTT assay.

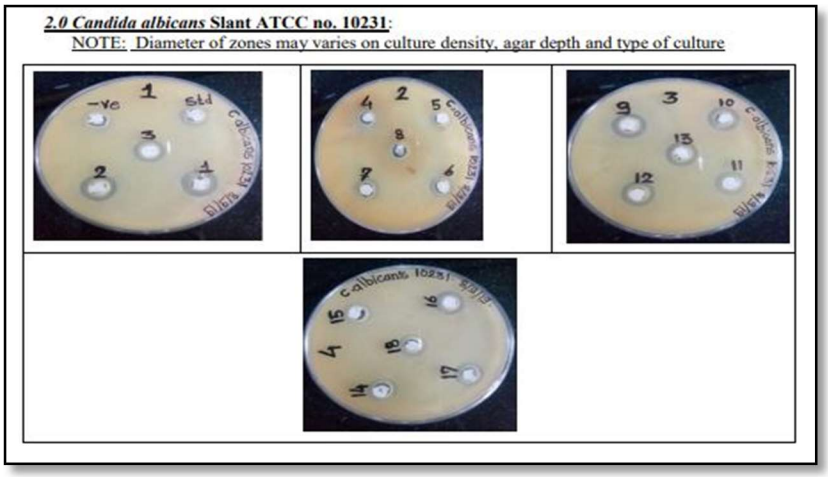
Sr.No.	**HL-2**	**ABS T_1**	**ABS T_2**	**ABS T_3**	**Mean O.D**	**%of cell viability**	**% of cell inhibition**	**Ic_{50}**
1	Control	0.312	0.311	0.313	0.312			79.11
2	200µg/ml	0.134	0.139	0.132	0.135	43.27	56.73	
3	400µg/ml	0.111	0.112	0.107	0.110	35.26	64.74	
4	600µg/ml	0.094	0.091	0.094	0.093	29.81	70.19	
5	800µg/ml	0.052	0.054	0.053	0.053	16.99	83.01	
6	1000µg/ml	0.024	0.021	0.026	0.023	07.38	92.62	

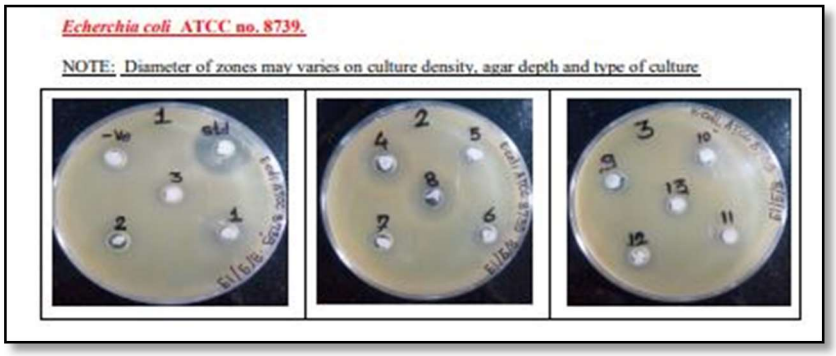
8.3 Effect of HL-3 against Hep G2 cell line (liver cancer cell line) by MTT assay.

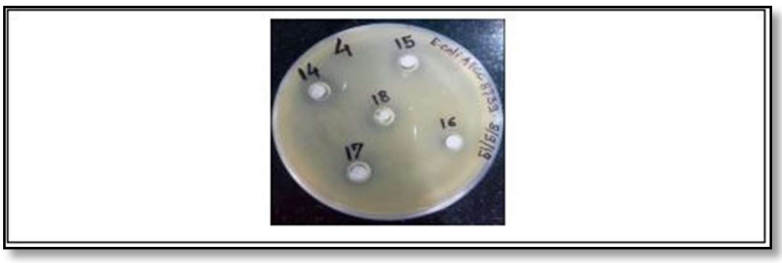
Sr.No	HL-3	ABS T_1	ABS T_2	ABS T_3	Mean O.D	%of cell viability	% of cell inhibition	Ic_{50}
1	Control	0.312	0.311	0.313	0.312			620.92
2	200µg/ml	0.222	0.213	0.201	0.212	67.95	32.05	
3	400µg/ml	0.196	0.195	0.191	0.194	62.18	37.82	
4	600µg/ml	0.181	0.182	0.179	0.180	57.70	42.30	
5	800µg/ml	0.131	0.134	0.131	0.132	42.31	57.69	
6	1000µg/ml	0.074	0.070	0.072	0.072	23.08	76.92	

Antimicrobial studies









Schiff base first	14.33	Significant Antimicrobial	14.90	Significant Antimicrobial	12.53	No antimicrobial
Schiff base 4	14.99	Significant Antimicrobial	14.52	Significant Antimicrobial	14.63	Significant Antimicrobial
Schiff base two	13.01	Weak antimicrobial	12.31	No antimicrobial	13.83	No 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

Remark –

1. **Gram Negative antibacterial complexes Bacterial (E. coli) – Indicated by GREEN**
2. **Gram positive antibacterial complexes Bacterial (S. aureus) – Indicated by BLUE**
3. Antifungal **complexes (Candida albicans) - Indicated by YELLOW**

Images of Antibacterial activity of HL-1 to HL-3

*Staphylococcus aureus*slant ATCC no. 6538

*Candida albicans*slant slant ATCC no. 10231.

Echerchia coli ATCC no.8739.

The Antibacterial activity of the Schiff bases were tested on *Staphylococcus aureus, Candida albicans and Echerchia coli.* The method used for anti-microbial activity was Agar Well-Diffusion method [Cup-Plate]. 10.00mg of Ampicillin dissolved dilute with 10ml DMSO(Dimethyl sulphoxide) in volumetric flask.1mg/ml the stock

solution was prepared and used to prepare its concentration .Streak of a loopful suspension of test organisms on two slant of pre incubated sabourauds dextrose agar. After incubating the slants at 20-25^{o}C for 72 hours in an incubator. After incubation picking up the growth from incubated slant and inoculating in 3 ml of saline solution and vortex was to prepare the uniform suspension.

Adjusting the optical density (O.D)of culture to approximately 60-70% O.D at 530 nm using sterile saline and calorimeter stored the test organism in refrigeration at 2-8 ^{o}C. After completion of incubation accurately measures the diameter of areas of the circular inhibition zones. The results are summarized in table 6 to 7.Schiff base (HL-1) is biologically active & its activity arise from the presence of imino group and chloro group which elucidating anti-microbial activity in biological systems.HL-1 shows significant anti-microbial activity against *Staphylococcus aureus (*Gram positive bacteria)and *Echerchia coli(*Gram negative bacteria).and weak significant against *Candida albicans*(Yeast).strain with respect to the standard Ampicillin.

The results obtained, anti-bacterial, activity of [SB-1] is found to be higher than that of [SB-2]and [SB-4] ligand, against the microorganism under identical experimental conditions.

Anti-Cancer Activity Studies:

All the synthesized Schiff bases were screened for their anti-cancer activity. Anti-cancer activity was measured In-Vitro cytotoxicity for the newly synthesized Schiff bases using Fluorouracil (5- FU,30µm)used as positive control by MTT Assay Method. Cells were incubated at a concentration of 1×10^{4} cells /ml in culture medium for 24 hours at 37 ^{o}C and 5 % CO_2.Control wells were incubated with DMSO (0.2% in PBS) and cell line. All samples were incubated in triplicate .Triplicate samples were analyzed by measuring the absorbance of each sample by micro plate reader at a wavelength of 550 nm.

IC 50 values of ligand SB-2 Hep G2 cells after treated for 24 Hr was less than that of ligand SB-1[HL-1]and SB-3 [HL-3].It results that ligand HL-2 possessed more potent inhibitory effect against the cancer cells(Liver cancer cell line Hep G-2).The results of anti-cancer activity of all synthesized compounds mentioned in table [8.1 to 8.3].Also

graphs shows percentage of cell viability and percentage of cell viability and percentage of cell inhibition.

5. CONCLUSION

The structures of the synthesized Schiff base ligands was confirmed by IR,^{1}H-NMR,^{13}C-NMR data and CHN analysis. The HL-1 to HL-3 are strongly active against *Staphylococcus aureus* and *Candida albicans &* weakly significant against *Escherchia coli.* Main core outcome & outcome of our work is that SB-2(HL-2) is highly active against liver cancer cell line Hep G2

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