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SYNTHESIS AND BIOLOGICAL EVALUATION OF SOME NEW 10-[2-SUBSTITUTEDIMINO-4-SUBSTITUTED AMINO)-1,3,5-DITHIAZINO] CHLOROPHENOTHIAZINES.

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Abstract

In recent times**,** a series of 10- [2-substitutedimino-4-substituted amino)-1,3,5-dithiazino] Chlorophenothiazines was synthesized by the interaction with substituted isocyanodichloride having 50% acetone-ethanol solvent by conventional method. This method has broad applications due to the efficacy of this approach makes it suitable for accelerating a variety of organic processes, resulting in increased yield, improved selectivity, and reduced production of by-products. As a result, this approach provides a simpler process for extracting the desired products, which are produced in a relatively pure state. Synthesized compounds are verified by elemental analysis, chemical characterization and spectral analysis like IR, NMR and Mass. All synthesized compounds (4a-d) showed moderate-to-significant anti-bacterial and anti-fungal activity. Compound 4d showed moderate activity against breast carcinoma (MCF-7) cell lines with IC_{50} values of 49.52 µM.

1.1 INTRODUCTION

In the field of organic chemistry, heterocyclic and heterocyclic compounds have enormous importance in various fields. It is reported that heterocyclic compounds containing drug having 1,3,5-dithiazino or 1,3,5-thiadiazino nucleus are widely used in medicinal and biochemical sciences[1-6]. Dithiazines are organosulfur heterocyclic compounds possessing a diverse range of biological activities including antibacterial[7-8], fungicidal[9], antiviral[10], anti-HIV[11], and anticancer activities[12].

Literature survey reveals that the phenyl isocyanide dichloride combines with 1-aryl-2,4-dithiobiurets in benzene under reflux to give 5-aryl-4-phenylimino-1,3,5-triazines[13], whereas the reactions with 1,3,5-trisubstituted 2,4-dithiobiurets in similar conditions afford substituted 1,3,5-dithiazines[14]. 1-Alkyl(aryl)-2,4-dithiobiurets were reacted with aldehydes and ketones in the presence of dry HCl[15] or ethyl chloroformate[16] to obtain hexahydro-1,3,5-triazine-4,6-dithiones, whereas 1-aroyl-2-bromoacetylenes with 2,4-dithiobiuret in benzene form 2-acylmethylene-4,6-diimino 5,6-dihydro-1,3,5-dithiazines[17]. The reactions of benzoyl acetylene, propionic acid, and methyl propionate with 2,4-dithiobiuret and 1-substituted and 1,5-disubstituted 2,4-dithiobiurets in glacial AcOH in the presence of HClO4 or BF3 were used to obtain 2-substituted 4-amino-6-imino-1,3,5-dithiazinium perchlorates or trifluoroborates, respectively[18].

The 2,4,6-trisubstituted 5,6-dihydro-l,3,5-dithiazines are obtained by the reaction of aliphatic and aromatic aldehydes with ammonia and hydrogen sulfide in alcohol at 0 ^{o}C[19]. The N-substituted 5,6-dihydro-l,3,5-dithiazines are obtained together with thioamides by the reaction of 2-substituted 1,3--dithietane with ethylamine in acetonitrile at 20 ^{o}C[20]. The 1-benzoyl- and l-(2-thenoyl)-2-bromoacetylenes react with dithiobiuret in glacial acetic acid or benzene at 20 ^{o}C with the formation of hydrobromides of 2-acylmethylene-4,6-diimino-5,6-dihydro- 1,3,5-dithiazines[21].

Sulphur act as an antibiotic having fewer side effects, excess use of sulphur may cause skin problems but when sulphur is present in ring its side effects decreases and potency of a molecule as well as biological activity increases. Considering this many researchers[22-25] synthesized such type of sulphur containing molecules. Some 1,3,5-dithiazino molecules showed anti-corrosive property against copper[26] and lubrication of oil[27]. By the known literature, isocyanodichloride are synthesized from various substituted isothiocyanates by passing chlorine gas[28].

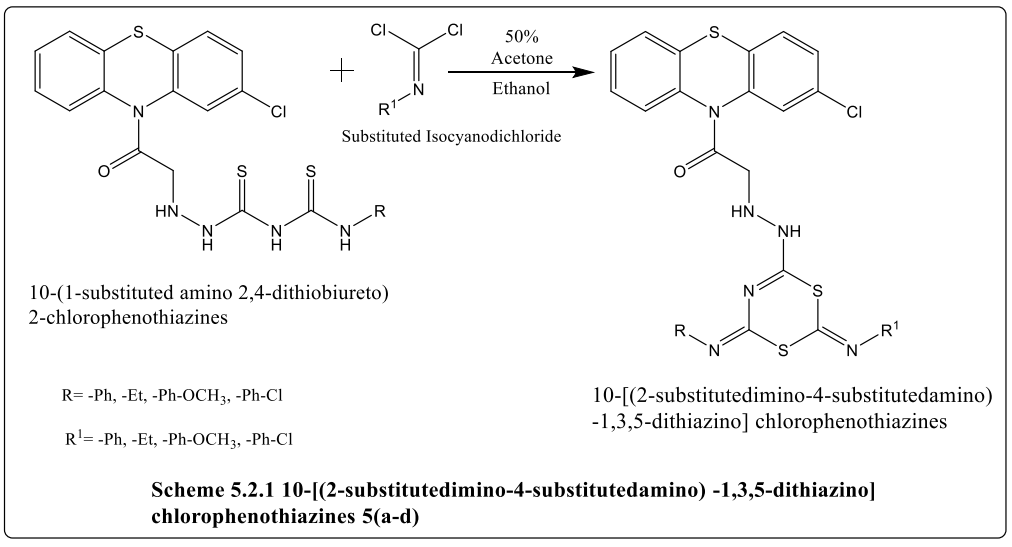
Recently a series of (2*E*)-1-[4-(2-substitutedimino-4-substitutedimino-1,3,5-dithiazino-6-yl)aminophenyl]-3-(3,4-dimethoxyphenyl)prop-2-en-1-one was synthesized by refluxing (2*E*)-1-

[4-(5-substituted-2,4-dithiobiureto)phenyl]-3-(3,4-dimethoxyphenyl) prop-2-en-1-one and alkyl/aryl isocyanodichlorides in acetone-ethanol medium[29]. Synthesis of 1-phenyl-3-[4-(2-substitutedimino-4-substitutedimino-1,3,5-dithiazino)-aminophenyl] prop-2-ene-1-ones from 1-phenyl-3-[4-(5-allyl-2,4-dithiobiureto) phenyl]-poro-2-ene-1-one and substituted isocyanodichloride had been reported[30].

As a part of research work presently undertaken in this laboratory in the synthesis of heterocycles, it was thought interesting to investigate the 10-[(2-substitutedimino-4-substitutedamino)-1,3,5-dithiazino] chlorophenothiazines. Phenothiazine nucleus in the same molecule which may increase biological activity.

1.2 PRESENT WORK

Literature survey showed that interaction of 10-(1-substituted amino 2,4-dithiobiureto) 2-chlorophenothiazines with various analogues of isocyanodichloride in 50% acetone-ethanol medium are still lacking, hence considering all these facts; it was thought to interest to synthesize a series of 10-[(2-substitutedimino-4-substitutedamino)-1,3,5-dithiazino] chlorophenothiazine [5(a-d)] **(Scheme 5.2.1)** and synthesized compounds were characterized by IR, ^{1}H NMR, and Mass spectra. Final compounds **[5(a-d)]** have been evaluated for their antibacterial, antioxidant and anti-cancer screening.



Scheme 5.2.1 10-[(2-substitutedimino-4-substitutedamino) -1,3,5-dithiazino] chlorophenothiazines 5(a-d)

1.3 RESULTS AND DISCUSSION

Formation of 10-substituted dithiazine derivatives of 2-chlorophenothiazine [5(a-d)] was confirmed based on elemental analysis, IR, NMR and Mass spectra. The IR spectra of compound show absorption in the range 3341–3333 cm^{-1} due to the presence N–H group, 1717-1714 cm^{-1} due to the presence C=O group, 933-924 cm^{-1} due to the presence C-S single bond group and 1120-1106 cm^{-1} due to the presence C-N bending vibration group.

The ^{1}H NMR spectrum of all synthesized compound showed a singlet at δ (8.77-8.76) due to the N-H protons, singlet at δ (4.26-3.35) due to the -CH2 protons, and multiplate at δ (7.74-6.65) due to the aromatic region, singlet at δ (9.69-11.1) due to the -NH-C=N protons, in compound 2b showed quartet δ (4.30-4.26), and δ triplet (1.29-1.26) due to the -CH_2, and -CH_3 protons, in compound 2c showed singlet δ (2.26) protons due to presence of - CH_3 group on aromatic.

Further evidence for the formation of compounds [5(a-d)] was obtained by recording the mass spectra. The mass spectrum of compounds [5(a-d)] showed a molecular ion peak at m/z 599.34, 582.25, 617.20 and 678.95 which is in conformity with the molecular formula.

All the synthesized compounds [5(a-d)] gives Lassigne's positive test for nitrogen and Sulphur. Desulphurization was not observed when warm with silver nitrate sodium plumbite solution indicating Sulphur is blocked in ring and formation of wine-red color by added sodium nitroprusside solution indicating that ketonic group is present.

R_f values of [5(a-d)] compounds were found to be 0.35, 0.37, 0.39, 0.37 by using Hexane+Ethyl acetate solvent 9:1 on silica Gel-G having layer thickness 0.3 mm.

All synthesized compounds screened their antimicrobial, antioxidant and anticancer activity showed moderate to significant activity.

1.4 EXPERIMENTAL SECTION

All chemicals were purchased from Aldrich and TCI, Mumbai (India), and were used without further purification. The melting points were determined in open capillaries using a Toshniwal melting point apparatus and are uncorrected. The IR spectra were recorded in the solid state as a KBr suspension on a Perkin– Elmer spectrum one FT-IR spectrophotometer and ^{1}H NMR spectra were obtained in DMSO-d^6 on a Brucker 400 MHz instrument using TMS as an internal standard (chemical shifts in δ, ppm), Mass spectra on a LCQ Advantage Thermo Finnigen spectrometer. Elemental analysis was performed on Carlo Erba 1108 analyzer.

General procedure for the preparation of 10-substituted dithiobiurate derivatives of 2-chlorophenothiazine [5(a-d)]:

Synthesis of 10-substituted dithiazine derivatives of 2-chlorophenothiazine [5(a-d)] using substituted isocyanodichloride. Equimolar quantities of substituted isocyanodichloride were added dropwise in the suspension of 10-(1-substituted amino 2,4-dithiobiureto) 2-chlorophenothiazines in 50% acetone-ethanol solvent and the reaction mixture was refluxed for about 3 hours at 80°C. During refluxing homogeneous reaction mixture was obtained and formation product was confirmed by TLC. It was then allowed to cool at room temperature then water was added to it and extracted with dichloromethane (DCM). The

organic layer was dried over anhydrous Na_2SO_4, and the solvent was evaporated to get certain product. All synthesized products were recrystallized by ethanol.

1.5 CHARACTERIZATION DATA

1.5.1: 2-(2-((2Z,4E)-2,4-bis(phenylimino)-4H-1,3,5-dithiazin-6-yl) hydrazinyl)-1-(2-chloro-10H-phenothiazin-10-yl) ethanone (5a)

M.F.: $C_{29}H_{21}ClN_6OS_3$, Yellow, Yield: 70% elemental analysis: calculated (Found) C: 57.94 (54.85), H: 3.52 (3.45), N: 13.98 (13.87), S: 16.00 (15.89) M.P.: 95-97 °C

IR (KBr): 3337.9 (-NH stretching), 3056.3 (Ar-H stretching), 2079.3 (S-C=N stretching), 1524.7 (Ar-C=C stretching), 1715.7 (C=O stretching), 924.9 (C-S Single bond bending), 1868.1 (-C=NH Stretching) and 1113.9 (C-N bending).

1H NMR (DMSO-*d6*, 400 MHz): δ 8.77 (s, 1H, -NH), 11.07 (s, 1H, -NH-N=), 6.65-7.03 (m, 7H, Ar-H) and 4.26 (s, carbonyl -CH_2).

LC-MS m/z (ES+): m/z: 599.3 $[M+H]^+$ Base peak: 355.3 (100%), 405.3 (50%)

1.5.2: 1-(2-chloro-10H-phenothiazin-10-yl)-2-(2-((2Z,4E)-4-((4-chlorophenyl) imino)-2-(ethylimino)-4H-1,3,5-dithiazin-6-yl) hydrazinyl) ethanone (5b)

M.F.: $C_{25}H_{20}Cl_2N_6OS_3$, Brown, Yield: 72% elemental analysis: calculated (Found) C: 51.10 (51.02), H: 3.58 (3.47), N: 15.56 (15.46), S: 21.38 (21.27)

M.P.: > 110-112 °C

IR (KBr): 3340.8 (-NH stretching), 3072.7 (Ar-H stretching), 2055.2 (S-C=N stretching), 1576.8 (Ar-C=C stretching), 1717.6 (C=O stretching), 926.8 (C-S Single bond bending), 1874.8 (-C=NH Stretching) and 1112.9 (C-N bending).

1H NMR (DMSO-*d6*, 400 MHz): δ 8.76 (s, 1H, -NH), Invisible (s, 1H, -NH-N=), 6.65-7.74 (m, 7H, Ar-H), 3.35 (s, carbonyl -CH_2). 4.302-4.248 (q, -CH_2), and 1.29-1.26 (t, -CH_3)

LC-MS m/z (ES+): m/z: 582.2 $[M+H]^+$ Base peak: 216.1 (100%), 223.0(80%)

1.5.3: 1-(2-chloro-10H-phenothiazin-10-yl)-2-(2-((2Z,4E)-4-(phenylimino)-2-(p-tolylimino)-4H-1,3,5-dithiazin-6-yl) hydrazinyl) ethanone (5c)

M.F.: $C_{30}H_{23}ClN_6OS_3$, Dark Brown, Yield: 70% elemental analysis: calculated (Found) C: 53.95 (53.86), H: 3.54 (3.48), N: 13.68 (13.58), S: 18.79 (18.76)

M.P.: 90-92 °C

IR (KBr):. 3333.1 (-NH stretching), 2987.8 (Ar-H stretching), 2074.5 (S-C=N stretching), 1519.9 (Ar-C=C stretching), 1714.7 (C=O stretching), 933.5 (C-S Single bond bending), 1883.5 (-C=NH Stretching) and 1120.6 (C-N bending).

1H NMR (DMSO-*d6*, 400 MHz): δ 8.76 (s, 1H, -NH), 9.69 (s, 1H, -NH-N=), 6.65-7.74 (m, 7H, Ar-H), 4.27 (s, carbonyl -CH_2), and 2.26 (s, -CH_3)

LC-MS m/z (ES+): m/z: 617.2 $[M+H]^+$ Base peak: 477.4 (100%), 478.4 (50%)

1.5.4: 2-(2-((2Z,4E)-2,4-bis((4-chlorophenyl) imino)-4H-1,3,5-dithiazin-6-yl) hydrazinyl)-1-(2-chloro-10H-phenothiazin-10-yl)ethanone (5d)

M.F.: $C_{29}H_{19}Cl_3N_6OS_3$, Brown, Yield: 75% elemental analysis: calculated (Found) C: 49.62 (49.57), H: 2.84 (2.82), N: 13.15 (13.05), S: 18.07 (17.97)

M.P.: 107-110 °C

IR (KBr): 3338.9 (-NH stretching), 3060.9 (Ar-H stretching), 2054.2 (S-C=N stretching), 1475.6 (Ar-C=C stretching), 1715.7 (C=O stretching), 928.7 (C-S Single bond bending), 1879.7 (-C=NH Stretching) and 1106.2 (C-N bending).

1H NMR (DMSO-*d6*, 400 MHz): δ 8.77 (s, 1H, -NH), 11.1 (s, 1H, -NH-N=), 6.65-7.03 (m, 7H, Ar-H) and 4.27 (s, carbonyl -CH_2).

LC-MS m/z (ES+): m/z: 678.9 $[M+H]^+$ Base peak: 561.08 (100%).

1.6 BIOLOGICAL SCREENING

1.6.1 Antibacterial and antifungal activity

All the synthesized compounds have been screened for antibacterial activity against Gram-positive bacteria Staphylococcus aureus, Gram-negative bacteria Escherichia coli and antifungal activity against Candida albicans by disc diffusion method using tetracycline (0.5 µg/µl) as standard for antibacterial and Clotrimazole (10 µg/µl) as standard for antifungal having two different concentrations 50 µl and 100 µl. Antimicrobial activity was evaluated by measuring zone of inhibition in mm by disc diffusion method

Disc Diffusion Method

Disc diffusion method the growth media (Muller Hinton Agar media for bacterial growth and potato dextrose agar media for fungal growth) were prepared and sterilized in autoclave at 15 psi for 15 min. These media were poured into petri plates under standard conditions and allowed to solidify. Standardized bacterial inoculum was spread uniformly over the surface of the medium using a sterile non-absorbent cotton swab, and finally, the swab was passed around the edge of the medium. The inoculated Petri plates were closed with the lid and allowed to dry at room temperature. The sample impregnated discs and standard discs were placed on the inoculated agar medium. All Petri plates were incubated at 37 ^oC for 24 h. After the incubation, diameter of zone of inhibition produced by the sample and standard was measured. The results of antimicrobial activity are tabulated in table 1.

Table 1: Antibacterial and Antifungal Activities

Compound	Zone of Inhibition in mm					
	Antibacterial Activity				**Antifungal Activity**	
	Staphylococcus aureus (Gram +)		*Escherichia coli* (Gram -)		*Candida albicans*	
Concentration ►	50µl	100µl	50µl	100µl	50µl	100µl
PTZ-5-1(a)	10	13	--	--	09	14
PTZ-5-2(c)	11	14	--	15	08	14
PTZ-5-2(d)	09	13	--	--	08	15
Tetracycline[a]	27		18		NA	
Chlotrimazole[b]	NA		NA		16	

‘‘--’’ Indicates bacteria are resistant to the compounds at concentration >100 µl. NA- Not Applicable
[a] Standard reference as antibacterial drug. [b] Standard reference as antifungal drug.

From above table 1 the synthesized compounds **(5a–d)** were screened in vitro antimicrobial activity. All the synthesized compounds have shown moderate to significant activity against pathogenic bacteria and fungi. Compounds **(5C)** showed significant antibacterial as well as antifungal activity against *S. aureus, E. Coli* and *C. albicans* (zone of inhibition 14, 15 and 14 mm, for 100µl concentration respectively) This may be due to the presence of electron-withdrawing phenyl group on ring nitrogen of a dithiazine moiety, it also enhance the activity due to the presence of phenothiazine could be another important reason for its antibacterial activity[31] (Upadhyay et al., 2009) and **(5a and 5d)** showed comparable activity as that of standard tetracycline drug against *S. aureus* (zone of inhibition 13 mm each, for 100µl). All the synthesized compounds **(5a–d)** are showed significant activity against *C. albicans* fungi comparable with standard clotrimazole drug due to the presence of electron withdrawing substituent are present on dithiazine moiety.

1.6.2 Antioxidant activity

DPPH radical scavenging method

Sample stock solutions (1.0 mg/ml) were diluted to appropriate final concentrations in ethanol. An ethanolic solution of 1 ml of DPPH (0.3 mM) was added to 0.5 ml of the compound and

allowed to react at room temperature in a dark place for 30 min. After 30 min, the absorbance values were measured at 518 nm. All the measurements were taken as a triplicate value. From the average of the absorbance values, lower absorbance of the reaction mixture indicates higher free radical scavenging activity[32] (Mensor et al., 2000). The DPPH radical scavenging capability was calculated using the following equation:

$$\% \text{ of inhibition} = (ABS_{control} - ABS_{test}) / ABS_{control} \text{ X } 100$$

The percentage antioxidant activity (% inhibition) was extrapolated against concentration of the compound and EC_{50} was determined graphically. The results are tabulated in Table 2 and represented in figs. 1, 2, 3.

Table 2: DPPH radical scavenging activity of some titled compounds

Compound	DPPH (EC_{50} µg/ml)
5a	77.87
5b	60.66
5c	31.82
Ascorbic acid	9.98

DPPH radical scavenging activity of compound 5a, 5b and 5c measured at 517 nm

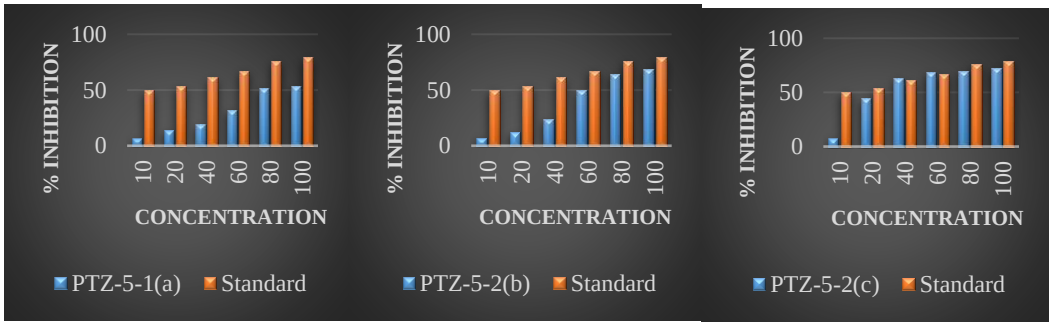


Fig. 1 **Fig. 2** **Fig. 3**

From the above table 2 and Fig. 1, 2 and 3 the antioxidant studies performed on three randomly selected samples against the standard ascorbic acid which revealed that three compounds (5a, 5b and 5c) showed moderate DPPH radical scavenging activity with EC_{50} values of 77.87, 60.66 and 31.82 µg/ml, respectively.

1.6.3 Anticancer Activity

Anticancer activity of samples determined by MTT assay method. The breast carcinoma (MCF-7) cell lines were obtained from the National Centre for Cell Science (NCCS), Pune. The cells were trypsinized and the cell count was adjusted to $5x10^5$ cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100µl

of the diluted cell suspension (50,000cells/well) was added. After 24 h, the supernatant was removed, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37oC for 24hrs in 5% CO2 atmosphere. After incubation the test solutions in the wells were discarded and 0.05mg

MTT was added to each well. The plates were incubated for 4 h at 37oC in 5% CO2 atmosphere. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line[33]. The results are tabulated in Table 3 and represented in Figs. 1, 2, 3 and4.

% of inhibition = 100 – ABS (Sample) / $ABS_{control}$ X 100

Table 3: In vitro cytotoxic activity against MCF-7 (breast carcinoma) cell lines of titled compound

MCF7 Cell line				
Compound Name	**Conc. µg/mL**	**O.D @ 570nm**	**% Inhibition**	**IC50 µg/mL**
Control	0	1.084	0	**56.76**
PTZ5_2(C)	7.81	1.000	7.71	
	15.62	0.845	22.01	
	31.25	0.582	46.29	
	62.5	0.487	55.05	
	125	0.362	66.64	
	250	0.171	84.22	
	500	0.082	92.48	
Control	0	1.224	0	**27.48**
PTZ5_2(d)	7.81	1.076	12.06	
	15.62	1.009	17.57	
	31.25	0.528	56.85	
	62.5	0.497	59.42	
	125	0.352	71.23	
	250	0.128	89.54	
	500	0.072	94.16	
Control	0	1.244	0	
	7.81	0.733	41.08	
	15.62	0.622	50.00	
	31.25	0.539	56.71	

Doxorubicin	62.5	0.516	58.52	**15.62**
	125	0.235	81.15	
	250	0.215	82.72	
	500	0.060	95.18	

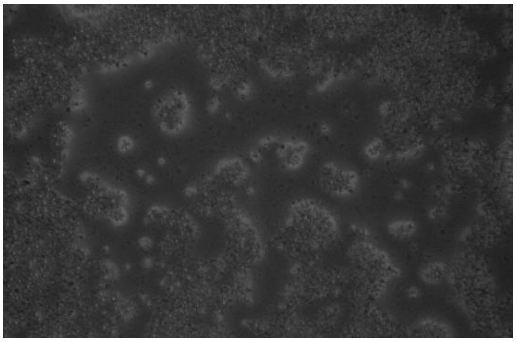


Fig. 1 Inhibition of MCF-7 by compound PTZ-5-2(c) (62.5M)

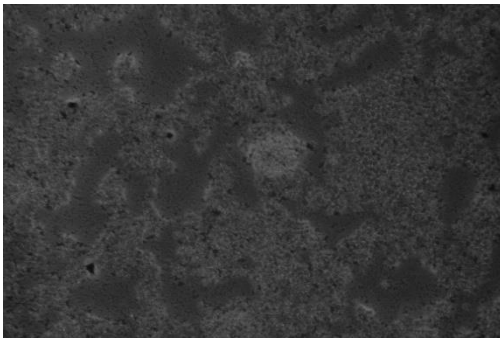


Fig. 2 Inhibition of MCF-7 by compound PTZ-5-2(c) (500M)

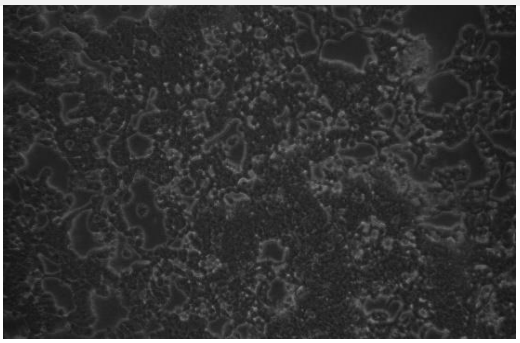


Fig. 3 Inhibition of MCF-7 by compound PTZ-5-2(d) (62.5M)

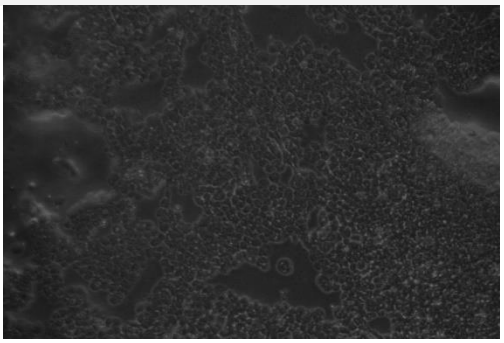


Fig. 4 Inhibition of MCF-7 by compound PTZ-5-2(d) (500M)

From the above table 3 and Fig. 1, 2, 3 and 4 the in vitro anticancer studies were performed randomly of titled compound using MTT assay against breast carcinoma (MCF-7) cell lines. The results indicated that the compounds 5d were found to have significant cytotoxic activity against MCF-7 cell line and then IC_{50} values were found to be 27.48 µg/mL comparable with standard doxorubicin drug due to the presence of electron withdrawing substituents are present on ring nitrogen of a dithiazine moiety. Compounds 5c showed moderate cytotoxic activity against MCF-7 cell line.

1.7 CONCLUSION

From all these studies, we have reported a facile and convenient route for the synthesis of 10- [2-substitutedimino-4-substituted amino)-1,3,5-dithiazino] Chlorophenothiazines [5(a-d)] having phenyl, ethyl, tolyl and p-chloro phenyl as a novel N-substituted 2-chlorophenothiazines. In the present work, simple workup procedure, short reaction time as

compared to other synthetic routes, no need of catalysts, and good to excellent yields of final products. All the synthesized compounds have been thoroughly characterized by IR, ^{1}H NMR, and Mass spectroscopy and screened for their anticancer, antioxidant and antimicrobial activities. All synthesized compounds (5a-d) showed moderate to significant antibacterial, antifungal and anticancer activity. The results obtained encouraged us to pursue further research in the synthesis of many derivatives of titled compounds to perform in vivo trials in experimental animals to broaden their pharmacological assessment and receptor interactions.

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