The Bioscan
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
19(2): S.I (1), 903-908, 2024 www.thebioscan.com

SYNTHESIS AND BIOLOGICAL EVALUATION OF SOME NEW 10-[(3-SUBSTITUTEDAMINO) 1, 2, 4- DITHIAZOLO] 2-CHLOROPHENOTHIAZINES

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DOI: https://doi.org/10.63001/tbs.2024.v19.i02.S.I(1).pp903-908

Received on:

22-09-2024

Accepted on:

20-10-2024

Published on:

25-11-2024

ABSTRACT

In recent times, a series of 10-[(3-substitutedamino) 1, 2, 4- dithiazolo] 2-chlorophenothiazines was synthesized by the oxidative cyclization with liquid bromine in chloroform medium 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.

INTRODUCTION

The chemistry of nitrogen-sulfur heteroatoms containing fragrant compounds is getting to be more prevalent as a zone of enquire about it. Phenothiazines are often regarded as highly versatile compounds from the point of view of biological activity. Since their discovery, new exploitable pharmacological properties have emerged from time to time; thus, they have a significant role in many areas of medicine and beyond. This is why phenothiazines are fundamental compounds in pharmacology. Phenothiazines are cheap and broadly accessible, and therefore phenothiazine derivatives show diverse biological activities[1-4] including antimicrobial[5], anticancer[6-7] and anti-inflammatory activities[8]. Great intrigued has emerged within the plan and blend of unused phenothiazines to evaluate their anticancer activities.

The dithiazole nucleus, which has a useful structure for further molecular exploration for the development of new derivatives with different biological activities, has received much attention in recent years[9]. Dithiazole nucleus are useful intermediates in medicinal chemistry like, pharmaceutical, industrial, biological, agricultural and medicinal fields[10-16].

Farghaly *et al*. synthesized a series of 1,3,4-thiadiazoles, evaluated their antimicrobial activity, and studied their structure activity relationship (SAR) towards some microorganisms. The results of this study revealed that this class of molecules holds promising activity[17].

Bhatia *et al*. synthesized various imidazo-thiadiazole derivatives and screened their antimicrobial activity. The results revealed that these molecules are active against *Shigella exneri*, *Staphylococcus aureus (S. aureus)* and *Candida albicans* bacterial strains[18].

Kaur *et al*. synthesized biphenyl imidazo[2,1-b] [1,3,4] thiadiazole derivatives and found that these molecules exhibit moderate to good activity when compared with standard antibiotics ampicillin and amphotericin B and screened the two derivatives for anticancer activity[19].

Gur *et al*. synthesized a series of 1,3,4-thiadiazole derivatives and reported that some of them had effective antioxidant properties[20].

Hakan S. S. *et al*. synthesized 1,3,4-Thiadiazole molecules were synthesized by the reaction of phenylthiosemicarbazide and methoxy cinnamic acid molecules in the presence of phosphorus oxychloride and investigate their antibacterial activities various bacteria strains such as *Enterobacter aerogenes, Escherichia coli*, *Salmonella kentucky, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus* and Gram positive such as *Staphylococcus aureus, Listeria monocytogenes, Enterococcus faecium, Enterococcus durans*, and *Staphylococcus aureus*. All the investigated compounds showed an inhibitory effect for the Staphylococcus epidermidis protein[21].

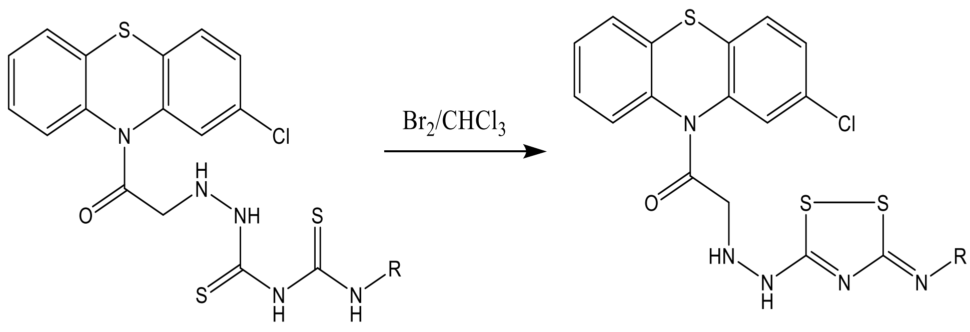
Recently in this laboratory a series of (2E)-1-[4-(3-substitutedimino-1,2,4-dithiazolo)aminophenyl]-3-(3,4-dimethoxyphenyl)prop-2-en-1-one synthesized by oxidative cyclisation of (2*E*)-1-[4-(5-substituted-2,4-dithiobiureto)phenyl]-3-(3,4-dimethoxyphenyl) prop-2-en-1-ones using liquid bromine in chloroform as an oxidizing agent[22]. 3-Substitutedimino-5-(4-pyridineimino) amino-1,2,4-dithiazoles were synthesized by oxidative cyclisation of 1-(4-pyridineimino)-5-substituteddithio biuretes[23].

Dabolkar and Ansari[24] successfully investigated oxidative cyclisation of cyanoamidino substituted thiocarbamide and N-substituted formidinothiocarbamides. Various researchers[25-28] studied oxidative cyclisation of some diformamidino thiocarbamides, substituted N-gluosides, cynoamidinothiocarbamides and thioglucosides to isolated 1,2,4-thiadiazoles, 1,3,4-thiadiazoles, 1,3,4-thadiazolines and 1,2,4-triazoles.

As a part of research work presently undertaken in this laboratory in the synthesis of heterocycles, it was thought interesting to investigate the cyclization of 10-(1-substituted amino 2,4-dithiobiureto) 2-chlorophenothiazines with liquid bromine in chloroform medium to obtain a novel series of 10-[(3-substitutedamino) 1, 2, 4- dithiazolo] 2-chlorophenothiazines which are hither to unknown.

1.2 PRESENT WORK

Literature survey showed that oxidative cyclization of 10-(1-substituted amino 2,4-dithiobiureto) 2-chlorophenothiazines with liquid bromine in chloroform medium are still lacking, hence considering all these facts; it was thought to interest to synthesize a series of 10-[(3-substitutedamino) 1, 2, 4- dithiazolo] 2-chlorophenothiazines [4(a-d)] (**Scheme 1.2.1)** by oxidative cyclization method using Br_2 in Liq. $CHCl_3$ and synthesized compounds were characterized by IR, ^{1}H NMR, and Mass spectra. Final compounds **[4(a-d)]** have been evaluated for their antibacterial, antioxidant and anti-cancer screening.



10-(1-substituted amino 2,4-dithiobiureto) 2-chlorophenothiazines

10-[(3-substitutedamino) 1, 2, 4- dithiazolo] 2-chlorophenothiazines

R= -Ph, -Et, -Ph-OCH_3, -Ph-Cl

R= -Ph, -Et, -Ph-OCH_3, -Ph-Cl

Scheme 1.2.1 10-[(3-substitutedamino) 1, 2, 4- dithiazolo] 2-chlorophenothiazines 4(a-d)

1.3 RESULTS AND DISCUSSION

Formation of 10-substituted dithiazole derivatives of 2-chlorophenothiazine [4(a-d)] was confirmed based on elemental analysis, IR, NMR and Mass spectra. The IR spectra of compound show absorption in the range 3341-3337 cm^{-1} due to the presence N-H group, 1714-1709 cm^{-1} due to the presence C=O group, 1477-1390 cm^{-1} due to the presence C=S group and 1102-1101 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 (4.28-4.10) due to the -CH2 protons, and multiplate at δ (7.74-6.65) due to the aromatic region, singlet at δ (10.97-11.17) due to the -NH-C=N protons, in compound 2b showed quartet δ (4.30-4.28), and δ triplet (1.29-1.26) due to the -CH_2, and -CH_3 protons, in compound 2c showed singlet δ (2.264) protons due to presence of - CH_3 group on aromatic.

Further evidence for the formation of compounds [4(a-d)] was obtained by recording the mass spectra. The mass spectrum of compounds [4(a-f)] showed a molecular ion peak at m/z 507.30, 452.0, 511.20 and 534.10 which is in conformity with the molecular formula.

All synthesized compounds 4(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[29-30] and formation of wine-red color by added sodium nitroprusside solution indicating that ketonic group is present.

R_f values of compounds 4(a-d) was found to be 0.40, 0.39, 0.41, 0.42 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 results.

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 [4(a-d)]:
Synthesis of 10-substituted dithiazole derivatives of 2-chlorophenothiazine [4(a-d)] using liquid bromine in presence of chloroform. In china dish a pest of 10-(1-substituted amino 2,4-dithiobiureto) 2-chlorophenothiazines was taken to it liquid bromine in chloroform was added with constant stirring. Initially the colour of bromine disappeared, addition was continued till colour of bromine persisted. The reaction mixture was allowed to stand for 4 hours and then on basification with dilute ammonium hydroxide 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: (Z)-1-(2-chloro-10H-phenothiazin-10-yl)-2-(2-(3-(phenylimino)-3H-1,2,4-dithiazol-5-yl) hydrazinyl) ethanone (4a)

M.F.: $C_{22}H_{16}ClN_5OS_3$, Pale Yellow, Yield: 75% elemental analysis: calculated (Found) C: 53.05 (52.98), H: 3.24 (3.15), N: 14.06 (13.98), S: 19.31(19.18) M.P.: 120-122 °C

IR (KBr):.3337.96 (-NH stretching), 3057.3 (Ar-H stretching), 1522.8 (-C=NH stretching), 1591.3 (Ar-C=C stretching), 1711.9 (C=O stretching), 923.9 (C-S Single bond stretching) and 1101.4 (N-C=S bending).

1H NMR (DMSO-*d6*, 400 MHz): δ 8.71 (s, 1H, -NH), 11.0 (s, 1H, -NH-N=), 6.65-7.03 (m, 7H, Ar-H) and 4.28 (s, carbonyl -CH_2).

LC-MS m/z (ES+): m/z: 507.30 $[M+H]^+$ Base peak: 228.25 (100%), 223.15 (80%)

1.5.2: (Z)-1-(2-chloro-10H-phenothiazin-10-yl)-2-(2-(3-(ethylimino)-3H-1,2,4-dithiazol-5-yl) hydrazinyl) ethanone (4b)

M.F.: $C_{18}H_{16}ClN_5OS_3$, Light Brown, Yield: 80% elemental analysis: calculated (Found) C: 48.04 (48.00), H: 3.58 (3.42), N: 15.56 (15.41), S: 21.38 (21.26) M.P.: 133-135 °C

IR (KBr):.3341.82 (-NH stretching), 3068.8 (Ar-H stretching), 1462.1 (-C=NH stretching), 1583.6 (Ar-C=C stretching), 1709.9 (C=O stretching), 927.8 (C-S Single bond stretching) and 1103.3 (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), 3.33 (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: 452 $[M+H]^+$ Base peak: 190.0 (100%), 441.0(70%)

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

M.F.: $C_{23}H_{18}ClN_5OS_3$, Dark Brown, Yield: 80% elemental analysis: calculated (Found) C: 53.95 (53.87), H: 3.54 (3.45), N: 13.68 (13.56), S: 18.79 (18.65) M.P.: 110-112 °C

IR (KBr):.3337.9 (-NH stretching), 3050.5 (Ar-H stretching), 1524.7 (-C=NH stretching), 1594.2 (Ar-C=C stretching), 1709.9 (C=O stretching), 930.6 (C-S Single bond stretching) and 1102.3 (C-N bending).

1H NMR (DMSO-*d6*, 400 MHz): δ 8.71 (s, 1H, -NH), 10.97 (s, 1H, -NH-N=), 6.65-7.74 (m, 7H, Ar-H), 4.28 (s, carbonyl $-CH_2$), and 2.26 (s, $-CH_3$)

LC-MS m/z (ES+): m/z: 511.2 $[M+H]^+$ Base peak: 330.35 (100%), 257.3 (60%)

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

M.F.: $C_{22}H_{15}Cl_2N_5OS_3$, Pale Yellow, Yield: 80% elemental analysis: calculated (Found) C: 49.62 (49.53), H: 2.84 (2.69), N: 13.15 (13.11), S: 18.07 (17.98) M.P.: 115-117 °C

IR (KBr):.3339.8 (-NH stretching), 3065.0 (Ar-H stretching), 1515.1 (-C=NH stretching), 1581.7 (Ar-C=C stretching), 1714.7 (C=O stretching), 930.6 (C-S Single bond stretching) and 1100.4 (C-N bending).

1H NMR (DMSO-*d6*, 400 MHz): δ 8.71 (s, 1H, -NH), 11.0 (s, 1H, -NH-N=), 6.65-7.03 (m, 7H, Ar-H) and 4.28 (s, carbonyl $-CH_2$).

LC-MS m/z (ES+): m/z: 534.10 $[M+H]^+$ Base peak: 223.10 (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 Dffusion 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-4-1(a)	07	11	--	07	10	14
PTZ-4-1(b)	06	10	--	06	12	13
PTZ-4-2(c)	--	11	--	--	11	15
PTZ-4-2(d)	09	12	--	--	12	14
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 **(4a-d)** were screened in vitro antimicrobial activity. All the synthesized compounds have shown moderate to significant activity against pathogenic bacteria and fungi. Compounds **(4d)** showed significant antibacterial as well as antifungal activity against *S. aureus* and *C. albicans* (zone of inhibition 12 and 14 mm, for 100µl concentration respectively) This may be due to the presence of electron-withdrawing chlorophenyl group on ring nitrogen of a dithiazole moiety, it also enhance the activity due to the presence of phenothiazine could be another important reason for its antibacterial activity[29] (Upadhyay et al., 2009) and **(4c)** showed comparable activity as that of standard tetracycline drug against *S. aureus* (zone of inhibition 11 mm, for 100µl). All the synthesized compounds **(4a-d)** are showed better activity against *C. albicans* fungi comparable with standard clotrimazole drug. The compounds **(4a)** and **(4b)** which showed equipotent activity against *S. aureus* (zone of inhibition 11 and 10 mm, for 100µl concentration respectively) due to the presence of phenyl and ethyl substituent are present on dithiazole 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[31] (Mensor et al., 2000). The DPPH radical scavenging capability was calculated using the following equation:

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

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)
4b	88.37
4c	111.58
4d	76.78
Ascorbic acid	9.98

DPPH radical scavenging activity of compound 4b, 4c and 4d measured at 517 nm

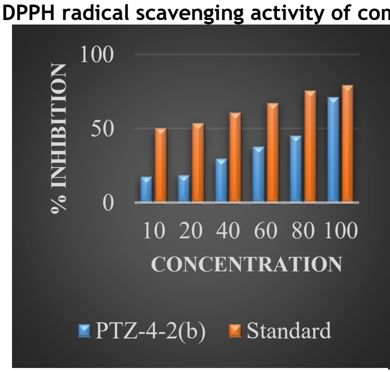


Fig. 1

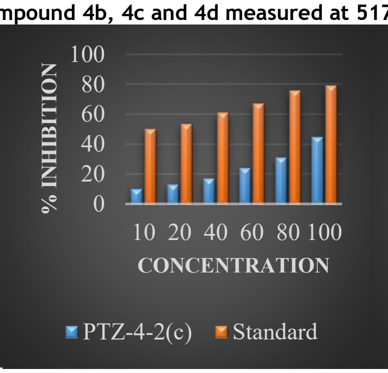


Fig. 2

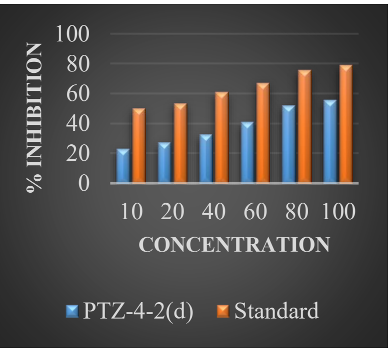


Fig. 3

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 (4b, 4c and 4d) showed moderate DPPH radical scavenging activity with EC_{50} values of 88, 111, 66 and 76 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 5×10^5 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[32]. The results are tabulated in Table 3 and represented in Figs. 1, 2, 3 and4.

$$\boxed{\% \text{ of inhibition} = 100 - \text{ABS (Sample)} / \text{ABS}_{control} \text{ X } 100}$$

es of titled compound

MCF7 Cell line				
Compound Name	**Conc. µg/mL**	**O.D @ 570nm**	**% Inhibition**	**IC50 µg/mL**
Control	0	0.973	0	**49.52**
PTZ 4d	7.81	0.944	2.98	
	15.62	0.913	6.22	
	31.25	0.650	33.20	
	62.5	0.359	63.10	
	125	0.225	76.93	
	250	0.121	87.56	
	500	0.072	92.60	
Control	0	1.244	0	**15.62**
Doxorubicin	7.81	0.733	41.08	
	15.62	0.622	50.00	
	31.25	0.539	56.71	
	62.5	0.516	58.52	
	125	0.235	81.15	
	250	0.215	82.72	
	500	0.060	95.18	

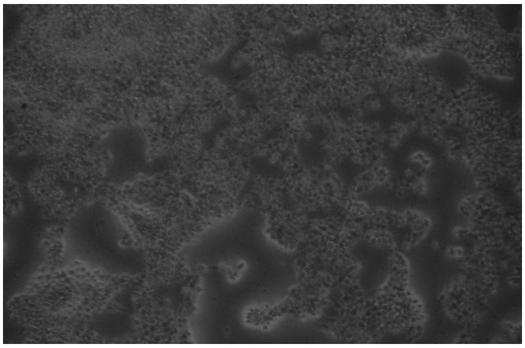


Fig. 1 Inhibition of MCF-7 by compound PTZ-4-2(d) (7.81M)

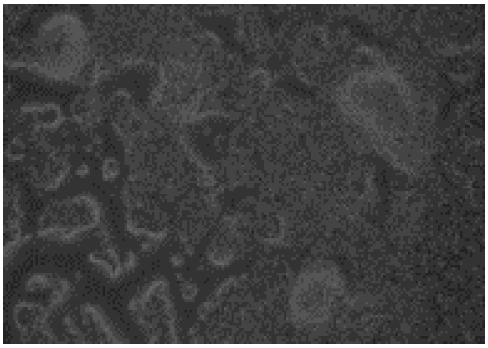


Fig. 2 Inhibition of MCF-7 by compound PTZ-4-2(d) (62.5M)

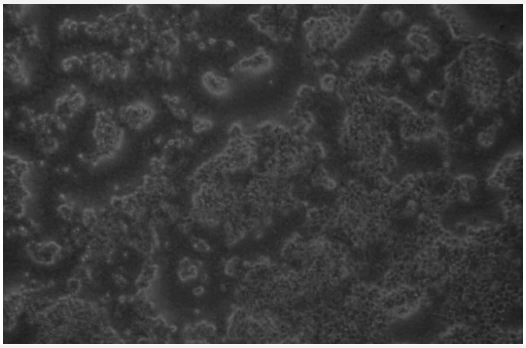


Fig. 3 Inhibition of MCF-7 by compound PTZ-4-2(d) (500M)

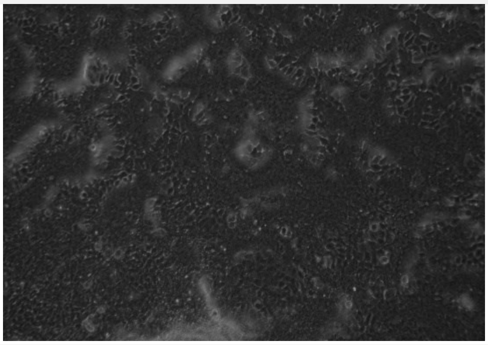


Fig. 4 Inhibition of MCF-7 by compound PTZ-4-2(d) (control)

From the above table 3 and Fig. 1, 2, 3 and 4 the in vitro anticancer studies were performed on only one titled compound using MTT assay against breast carcinoma (MCF-7) cell lines. The results indicated that the compounds 4d were found to have moderate cytotoxic activity against MCF-7 cell line and then IC_{50} values were found to be 49.52 μg/mL comparable with standard doxorubicin drug.

CONCLUSION

From all these studies, we have reported a facile and convenient route for the synthesis of 10-[(3-substitutedamino) 1, 2, 4-dithiazolo] 2-chlorophenothiazines [4(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 routs, 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 (4a-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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