Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(4):988-994

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Synthesis and anticancer activity of some new *N*'-[(3-Substituted alkyl/aryl)-4-oxo-1,3-thiazolidin-2-ylidene]-4-hydroxybenzohydrazide derivatives

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ABSTRACT

A novel series of N'-[(3-Substituted alkyl/aryl)-4-oxo-1,3-thiazolidin-2-ylidene]-4-hydroxybenzohydrazide (4.a-4.m) compounds were synthesised. The compounds were tested for its toxicity (in vivo). The cut off LD_{50} were found ≥ 1000 mg/kg. Anticancer activity of these compounds was performed In vitro by cell proliferation assay using 3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) staining method. Cell lines (A-549), Adeno carcinomic human alveolar basal epithelial cell line is used for screening. The compds, (6.h), (6.i), (6.k), (6.l) and (6.m), showed significant anticancer activity as percent cell lysis <75 to 100. Other compounds also exhibited promising anticancer activity.

Key words: Anticancer activity, Hydroxybenzohydrazide, Thiazolidin, MTT assay

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INTRODUCTION

The dramatically rising prevalence past few years cancer has become a serious health care problem. Cancer is uncontrolled growth of abnormal cells in the body. Cancerous cell are malignant cells. Normal cell multiply when the body needs them & die when the body does not need them [1-3].

The *p*-hydroxybenzohydrazide moiety revealed to be good candidate for anticancer activity. Hence taking hint from this it was observed that wide varieties of aliphatic/aromatic substitutions were possible on the moiety. The thiazolidine ring is helpful for imparting the anticancer activity to the compounds containing 4-hydroxybenzohydrazide ring. Further emphasis is given for preparation of possible derivatives from these moieties and screened for its biological activity [4-8].

EXPERIMENTAL SECTION

2.1 Materials and methods:

2.1.1 Instrumentation and Chemicals:

All the chemicals were of Alfa Aesar (UK), E. Merck laboratory grade. The percentage yields are based upon the products obtained after purification through crystallization. The melting points of compounds were determined in open capillary method and were uncorrected. The melting points are mentioned and are in centigrade. Silica gel G plates (activated at 100°C, 30 min) were used for thin layer chromatography and were developed in iodine vapor chamber. The R_f value is reported for better comparable solvent system. The IR spectra of synthesized compounds were recorded using KBr pellets on FTIR-8400 S, Shimadzu Marce and are in cm^{-1}. The ^1H NMR spectra ($CDCl_3$) were recorded on BRUKER AVANCE II 400 NMR Spectrometer (chemical shift in δ ppm) and Mass spectra were recorded on JEOL-Accu TOF JMS-T100LC DART-MS spectrometer.

2.1.2 Anticancer Activity:

Anticancer activity was carried out *In vitro* by cell proliferation assay using 3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) staining method. Cell lines (A-549), Adeno carcinomic human alveolar basal epithelial cell line is used for screening.

The MTT assay is colorimetric assays for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. A main application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth [9-12].

2.2 Chemistry:

The present study describes the synthesis and anticancer activity of some *N*'-[(3-Substituted alkyl/aryl)-4-oxo-1,3-thiazolidin-2-ylidene]-4-hydroxybenzohydrazide derivatives. A series of 13 new derivatives (4.a-4.m) were synthesized in satisfactory yields (67–94%) as illustrated in synthetic scheme and their structures were characterized by spectral data [13-20].

Synthetic Scheme

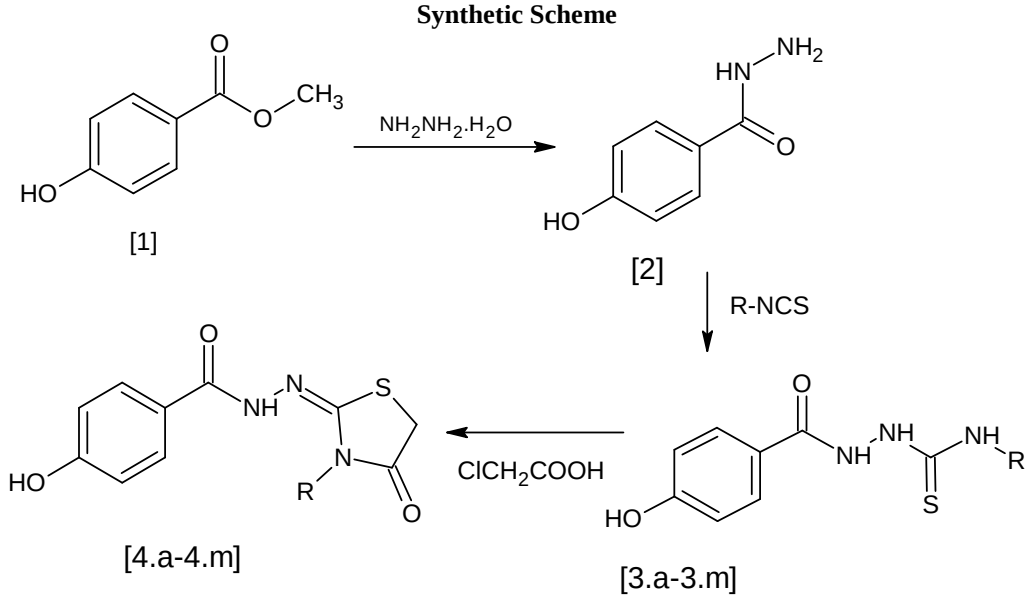
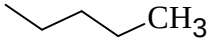
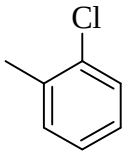
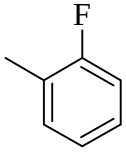
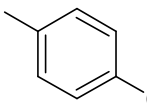
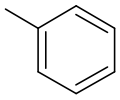
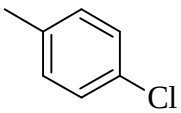
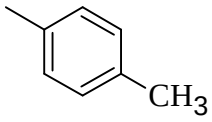
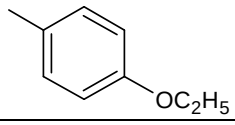
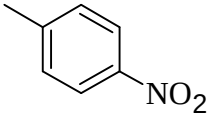
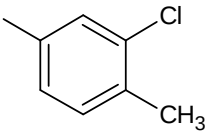
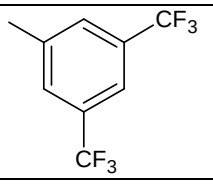
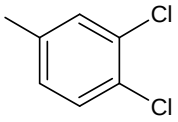
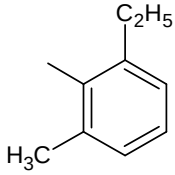


Table 1 Substitutions of derivatives

Compd no.		R = Structure and Name	
3.a	4.a		butyl
3.c	4.c		2-chlorophenyl
3.e	4.e		2-flurophenyl
3.g	4.g		4-hydroxy-phenyl

Compd no.		R = Structure and Name	
3.b	4.b		phenyl
3.d	4.d		4-chlorophenyl
3.f	4.f		4-methylphenyl
3.h	4.h		4-ethoxyphenyl

3.i	4.i		4-nitrophenyl
3.k	4.k		3-chloro-4-methylphenyl
3.m	4.m		3,5-bis-(trifluoromethyl)phenyl

3.j	4.j		3,4-dichloro-phenyl
3.l	4.l		2-ethyl-6-methyl phenyl

2.2.1 Procedure for synthesis of compd (2)

The mixture of **(1)** (1 g, 0.01 mol) and hydrazine hydrate 99% (30mL, 6 mol) was refluxed for 20 h. The reaction mixture was cooled at 4-5^0. The solid of (2) were filtered and washed with cold water. The product **(2)** was dried and recrystallized from ethanol.

2.2.2 Generalised procedure for Synthesis of *N*-substituted-2-[(4-hydroxyphenyl)carbonyl] hydrazine carbothioamide (3.a-3.m).

To a solution of compound (2) (1.5g 0.01 mol) in ethanol (50 mL), substituted- isothiocynate (0.01 mol) was added. The reaction mixture was refluxed for 12-17 h. Excess solvent was removed under vacuum. The residue was washed with diethyl ether and recrystallized to obtain the product (3.a-3.m)

2.2.3 Generalised procedure for Synthesis of *N*'-[(3-Substituted alkyl/aryl)-4-oxo-1,3-thiazolidin-2-ylidene]-4-hydroxybenzohydrazide (4.a-4.m).

A mixture of compound (3.a-3m) (0.01 mol), chloroacetic acid (0.01 mol) and sodium acetate (0.2 mol) in ethanol (60 mL) was heated under reflux for 10 h. The mixture was cooled and diluted with enough water to develop turbidity. The mixture was left overnight for complete separation of the product. The separated product was filtered, dried gave the corresponding product (4.a-4.m).

RESULTS AD DISCUSSION

3.1 Synthesis and physicochemical characterization

***N*'-(3-butyl-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydroxybenzo-hydrazide (4.a)**

Yield: 2.2 g (85%); mp 206-207^0 (methanol); R_f 0.59 (acetonitrile:methanol 1:1); IR (KBr) cm^{-1}: 1732 (C=O stretching) ; ^{1}H NMR ($CDCl_3$): δ 0.95 (t, 3H, butyl CH_3), 1.28 (m, 2H, butyl CH_2), 1.51 (m, 2H, butyl CH_2), 3.53 (m, 2H, butyl CH_2), 3.01 (s, 2H, thiazolidine CH_2), 5.35 (s,1H, Ar-OH), 7.73 (s, 1H, CONH); EIMS (m/z, 100%): 307 ([M+2], 100%). Anal. $C_{14}H_{17}N_3O_3S$; C, 53.91/53.91; H,6.35 /6.41; N,15.62/15.72.

***N*'-(3-phenyl-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydroxybenzo-hydrazide (4.b)**

Yield: 2.1 g (84%); mp 256-257^0 (methanol); R_f 0.66 (acetonitrile:methanol 1:1); IR (KBr) cm^{-1}: 1732 (C=O stretching); ^{1}H NMR ($CDCl_3$): δ 3.04 (s, 2H, thiazolidine CH_2), 5.35 (s,1H, Ar-OH), 7.73 (s, 1H, CONH), 6.38-7.85 (m, 9H, Ar-H); EIMS (m/z, 100%): 327 ([M+2], 100%). Anal. $C_{16}H_{13}N_3O_3S$; C,58.51 /58.52; H, 4.55/4.56; N, 14.52/14.62.

***N*'-[3-(2-chlorophenyl)]-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydro-xybenzohydrazide (4.c)**

Yield: 2.2 g (71%); mp 255-256^0 (methanol); R_f 0.6 (acetonitrile:methanol 1:1); IR (KBr) cm^{-1}: 1748 (C=O stretching); ^{1}H NMR ($CDCl_3$): δ 3.71 (s, 2H, thiazolidine CH_2), 5.22 (s,1H, Ar-OH), 7.87 (s,1H, CONH), 6.33-7.86 (m, 7H, Ar-H); EIMS (m/z, 100%): 379 (M+2), 100%). Anal. $C_{16}H_{11}ClN_3O_3S$;C, 47.21/47.20; H,3.11 /3.11; N,11.76/11.80.

***N*'-[3-(4-chlorophenyl)]-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydro-xybenzohydrazide (4.d)**

Yield: 2.6 g (75%); mp 245-246^0 (methanol); R_f 0.56 (acetonitrile:methanol 1:1); IR (KBr) cm^{-1}: 1743 (C=O stretching); ^{1}H NMR ($CDCl_3$): δ 3.76 (s, 2H, thiazolidine CH_2), 5.28 (s,1H, Ar-OH), 7.82 (s,1H, CONH), 6.38-7.85 (m, 7H, Ar-H); EIMS (m/z, 100%): 379 (M+2), 100%). Anal. $C_{16}H_{11}ClN_3O_3S$;C, 47.21/47.20; H,3.11 /3.11; N,11.76/11.80.

***N*'-[3-(2-fluorophenyl)]-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydro-xybenzohydrazide (4.e)**
Yield: 2.7 g (89%); mp 223-224^0 (methanol) ; R_f 0.7 (acetonitrile:methanol 1:1); IR (KBr) cm^{-1}: 1792 (C=O stretching), 1045 (C-F Stretching); ^{1}H NMR ($CDCl_3$): δ 3.76 (s, 2H, thiazolidine CH_2), 5.30 (s,1H, Ar-OH), 8.04 (s,1H, CONH), 6.38-7.85 (m,8H,Ar-H); EIMS (m/z, 100%): 372 ([M+2], 100%). Anal. $C_{16}H_{12}FN_3O_3S$; C,55.05/55.07; H, 3.95/3.96; N,13.78/13.76.

***N*'-[3-(4-methylphenyl)]-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydro-xybenzohydrazide (4.f)**
Yield: 2.6 g (87%); mp 237-238^0 (methanol); R_f 0.61 (acetonitrile:methanol 1:1), IR (KBr) cm^{-1}: 1773 (C=O stretching) ; ^{1}H NMR ($CDCl_3$): δ 2.11 (s, 3H, CH_3), 3.16 (s, 2H, thiazolidine CH_2), 5.34 (s,1H, Ar-OH),7.62 (s, 1H, CONH), 6.48-7.85 (m,7H,Ar-H); EIMS (m/z, 100%): 355 ([M+2], 100%). Anal. $C_{17}H_{15}N_3O_3S$; C, 60.91/60.93; H, 5.43/5.43; N,13.30/13.32.

***N*'-[3-(4-hydroxyphenyl)]-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydroxybenzohydrazide (4.g)**
Yield: 2.6 g (84%); mp 220-221^0 (methanol); R_f 0.61 (acetonitrile:methanol 1:1), IR (KBr) cm^{-1}: 1773 (C=O stretching); ^{1}H NMR ($CDCl_3$): δ 3.73 (s, 6H, OCH_3), 3.76 (s, 2H, thiazolidine CH_2), 5.41 (s,1H, Ar-OH),7.82 (s, 1H, CONH), 6.48-7.85 (m, 7H, Ar-H); EIMS (m/z, 100%): 379 ([M+2], 100%). Anal. $C_{16}H_{12}N_3O_4S$; C, 55.31 /55.32; H,4.90/4.93; N,12.06/12.10.

***N*'-[3-(4-ethoxyphenyl)]-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydro-xybenzohydrazide (4.h)**
Yield: 2.6 g (87%); mp 221-222^0 (methanol); R_f 0.61 (acetonitrile:methanol 1:1), IR (KBr) cm^{-1}: 1773 (C=O stretching) ; ^{1}H NMR ($CDCl_3$): δ 2.11 (s, 3H, CH_3), 3.16 (s, 2H, thiazolidine CH_2), 5.34 (s,1H, Ar-OH),7.62 (s, 1H, CONH), 6.48-7.85 (m,7H,Ar-H); EIMS (m/z, 100%): 362 ([M+2], 100%). Anal. $C_{18}H_{16}N_3O_4S$; C, 60.91 /60.93; H, 5.43/5.43; N,13.30/13.32.

***N*'-[3-(4-nitrophenyl)]-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydro-xybenzohydrazide (4.i)**
Yield: 1.6 g (52%); mp 221-222^0 (methanol); R_f 0.56 (acetonitrile:methanol 1:1); IR (KBr) cm^{-1}: 1731 (C=O stretching),1555 (NO_2 stretching) ; ^{1}H NMR ($CDCl_3$): δ 3.98 (s, 2H, thiazolidine CH_2), 5.23 (s,1H, Ar-OH), 7.70 (s, 1H, CONH), 6.38-7.85 (m, 8H, Ar-H); EIMS (m/z, 100%): 372 ([M+2], 100%). Anal. $C_{16}H_{12}N_4O_5S$; C,50.51 /50.60; H,3.55/3.64; N,16.82/16.86.

***N*'-[3-(3,4-dichlorophenyl)]-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydroxybenzohydrazide (4.j)**
Yield: 2.6 g (75%); mp 232-233^0 (methanol); R_f 0.56 (acetonitrile:methanol 1:1); IR (KBr) cm^{-1}: 1743 (C=O stretching); ^{1}H NMR ($CDCl_3$): δ 3.76 (s, 2H, thiazolidine CH_2), 5.28 (s,1H, Ar-OH), 7.82 (s,1H, CONH), 6.38-7.85 (m, 7H, Ar-H); EIMS (m/z, 100%): 396 (M+2), 100%). Anal. $C_{16}H_{11}Cl_2N_3O_3S$;C, 47.21/47.20; H,3.11 /3.11; N,11.76/11.80.

***N*'-[3-(3-chloro-4-methylphenyl)]-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydroxybenzohydrazide (4.k)**
Yield: 2.6 g (87%); mp 217-218^0 (methanol); R_f 0.61 (acetonitrile:methanol 1:1), IR (KBr) cm^{-1}: 1773 (C=O stretching) ; ^{1}H NMR ($CDCl_3$): δ 2.11 (s, 3H, CH_3), 3.16 (s, 2H, thiazolidine CH_2), 5.34 (s,1H, Ar-OH),7.62 (s, 1H, CONH), 6.48-7.85 (m,7H,Ar-H); EIMS (m/z, 100%): 364 ([M+2], 100%). Anal. $C_{17}H_{15}$ Cl N_3O_3S; C, 60.91 /60.93; H, 5.43/5.43; N,13.30/13.32.

***N*'-[3-(2-ehtyl-6-methylphenyl)]-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydroxybenzohydrazide (4.l)**
Yield: 2.6 g (87%); mp 217-218^0 (methanol); R_f 0.66 (acetonitrile:methanol 1:1), IR (KBr) cm^{-1}: 1767 (C=O stretching) ; ^{1}H NMR ($CDCl_3$): δ 2.14 (s, 3H, CH_3), 3.14 (s, 2H, thiazolidine CH_2), 5.37 (s,1H, Ar-OH),7.67 (s, 1H, CONH), 6.51-7.89 (m,7H,Ar-H); EIMS (m/z, 100%): 363 ([M+2], 100%). Anal. $C_{19}H_{19}N_3O_3S$; C, 60.91 /60.93; H, 5.43/5.43; N,13.30/13.32.

***N*'-{3-[3,5-bis(trifluoromethyl)phenyl]}-4-oxo-1,3-thiazolidin-2-ylidene)-4-hydroxybenzohydrazide (4.m)**
Yield: 2.4 g (78%); mp 219-220^0 (methanol); R_f 0.56 (acetonitrile:methanol 1:1); IR (KBr) cm^{-1}: 1773 (C=O stretching); ^{1}H NMR ($CDCl_3$): δ 3.06 (s, 2H, thiazolidine CH_2), 5.23 (s,1H, Ar-OH), 7.82 (s,1H, CONH), 6.38-7.85 (m, 7H, Ar-H); EIMS (m/z, 100%): 411 (M+2), 100%). Anal. $C_{18}H_{11}F_6N_3O_3S$; C,52.01/52.01; H,3.41 /3.43; N,13.00/13.00.

3.2 Biological Activity

3.2.1 Acute toxicity study (LD_{50} determination)

The cut off LD50 level were determined as per the Guidelines of Organization for Economic Co-operation and Development (OECD).

The LD50 values of synthesized compounds were determined. However, few compounds from Schemes were tested. The compounds which having substitutions like nitro, fluro, chloro were tested for its toxicity (in vivo).

The compounds were administered orally to pairs of mice. The treated mice were observed continuously for two hours and then occasionally for further four hours, and finally overnight mortality recorded. The dose killing one out of three mice given a very approximately LD50. The final LD50 value was determined and is shown in Table.

Table 2 Acute Toxicity studies

Compd No	Cut off LD_{50} (mg)	Compd No	Cut off LD_{50} (mg)
4.c	1000	15.c	1000
4.d	1000	16.c	2000
4.e	1000	17.c	1000
4.i	1000	18.c	2000
4.j	2000	31.g	1000
4.k	2000	32.g	2000
4.m	2000	34.g	2000

3.2.2 Anticancer Activity:

Anticancer Activity by Cell proliferation assay (MTT assay)

MTT solution is prepared by 10 mg in 10 ml of Hank's balanced solution.It is specific for the cell line were maintained in 96 wells micro titer plate containing MEM media supplemented with 10% heat inactivated fetal calf serum (FCS), containing 5% of mixture of Gentamycin, Penicillin (100 Units/ ml) and Streptomycin (100 µg/ml) in presence of 5% CO_2 at 37ºC for 3-4 days.

After 3-4 days remove the supernatant and replace MEM media with Hank's balanced solution supplemented with Gentamycin, Penicillin and Streptomycin. Incubate overnight. *Invitro* growth inhibition effect of test compound was assessed by calorimetric or spectrophotometric determination of conversion of MTT into "Formazan blue" by living cells. Remove the supernatant from the plate and add fresh Hank's balanced salt solution and treated with different concentration of extract or compound appropriately diluted with DMSO. Control group contains only DMSO. After 24 h incubation at 37ºC in a humidified atmosphere of 5% CO_2, the medium was replaced with MTT solution (100µl, 1mg per ml in sterile Hank's balanced solution) for further 4h incubation. The supernatant carefully aspirated, the precipitated crystals of "Formazan blue' were solubilised by adding DMSO (200µl) and optical density was measured at wavelength of 570 nm.

Cell lines maintained in appropriate conditions were seeded in 96 well plates and treated with different concentrations of the test samples and incubated at 37 °C, 5% CO_2 for 96 hours. MTT reagent was added to the wells and incubated for 4 hours; the dark blue formazan product formed by the cells was dissolved in DMSO under a safety cabinet and read at 492nm. Percentage inhibitions were calculated using the following formula and the concentrations of test drug needed to inhibit cell growth by 50 % (An IC50) value is generated from % growth inhibition for cell line.

The result were represents the mean of three readings. The concentration at which the OD of treated cells was reduced by 50% with respect to the untreated control.

By using Formula

$$\text{Surviving cell (\%)} = \frac{\text{Mean OD of Test Comp}}{\text{Mean OD at Control}} \times 100$$

Table 3 Percent cell lysis

Compd	Concentration (µG)					
	10		20		30	
	O.D. at 492nm	% cell lysis	O.D. at 492nm	% cell lysis	O.D. at 492nm	% cell lysis
4.a	0.523	-	0.547		0.588	-
4.b	0.520	-	0.544	-	0.576	-
4.c	0.522	-	0.552	-	0.575	-
4.d	0.523	-	0.561	-	0.587	-
4.e	0.532	-	0.549	-	0.586	-
4.f	0.526	-	0.542	-	0.581	-
4.g	0.524	-	0.541	-	0.572	-
4.h	0.658	<75	0.747	<75	0.911	>75
4.i	0.635	<75	0.752	<75	0.864	>75
4.j	0.536	-	0.562	-	0.585	-
4.k	0.728	<75	0.816	>75	0.992	100
4.l	0.744	<75	0.826	>75	1.123	100
4.m	0.739	<75	0.865	>75	1.157	100
Control	0.545	-	0.545	-	0.545	-

CONCLUSION

The compds which are having substitutions like nitro, fluro, difluro were tested for its toxicity *(in vivo*) using OECD guidelines No.423. The cut off LD_{50} were found ≥ 1000 mg/kg.

The anticancer activity was carried out *In vitro* by cell proliferation assay using 3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) staining method. In this method study was performed on human cell line (A-549), Adeno carcinomic human alveolar basal epithelial cell line, deposited by M. Liber. The compounds, (6.h), (6.i), (6.k), (6.l) and (6.m), showed percent cell lysis <75 to 100.

It can be overall concluded that the compounds in Scheme having thiazoline moiety with flurophenyl/dichlorophenyl/difluorophenyl possess better anticancer activity. Moreover, position 3 and 4 of thiazoline seems to be better place for substitution for biological activity. Further, the phenyl moiety can be substituted with methyl/methoxy/fluoro/ chloro etc. at para position. It was observed that electron withdrawing or electron donating substitution showed better activity.

Acknowledgements:
Authors are thankful to All India Council for technical Education (AICTE) for their financial assistance under the Research Promotion Scheme F. No.: 8023/BOR/RID/RPS-190/2008-09.

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