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SYNTHESIS OF SOME NEW DERIVATIVES OF COUMARIN CONTAINING PYRAZOLINE AND INVESTIGATION AGAINST HUMAN LUNG CANCER CELL LINE

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ABSTRACT: Synthesis of some new derivatives of coumarin containing pyrazoline derivatives, being used as against human lung cancer cell line, 3-acetyl coumarin (I) was prepared by Knoevenagel condensation of salicyladehyde with ethyl acetoacetate in presence of piperidine. A series of 3-[(2E)-3-substituted-prop-2-enoyl]-2H-chromen-2-one derivatives (2a-h) were prepared by Claisen-Schmidt condensation of 3-acetyl coumarin with aromatic aldehydes in the presence of piperidine / n- butanol. Treatment of 3-substituted cinnamoyl coumarin with hydrazine hydrate in the presence of ethanol gave [5-substitutedphenyl]-4, 5-dihydro-1H-pyrazol-3-yl]-2H-chromen-2-one (3a-h). Title compound were synthesized and the structures of newly synthesized compounds were confirmed by IR and ^{1}H-NMR spectroscopy. All the synthesized compounds were tested for their anticancer activities using SRB assay. The anticancer activity revels that some of the synthesized compounds possesses moderate anticancer activity.

KEY WORDS: Pyrazoline, aromatic aldehyde, anticancer activity, SRB assay.

INTRODUCTION: Coumarin compounds containing nitrogen and oxygen have received considerable attention due to their wide range of pharmacological activity [1].
Natural, semi synthetic and synthetic coumarins possess a protuberant place in drug research. Their utility stimulated the development of new synthetic routes for the preparation of coumarin containing pyrazoline derivatives. Moreover, coumarins have developed a special place in heterocyclic field because of their various activities such as antimalerial [2], anticonvulsant [3], anti-inflammatory [4], antioxidant [5], cytotoxic [5], anti-HIV [6] and anti-microbial [7].
Pyrazolines have played a crucial role in the development of theory in heterocyclic chemistry and also are extensively useful in organic chemistry. Due to interesting activity of various substituted pyrazolines as biological agents considerable attention has been focused on this paper. The pyrazolines can be effectively utilized as anti-malerial [2], anticonvulsant [8], antidepressant [8], antiepileptic [9], antidiabetic [10], antioxidant [11], anticancer [12], antimicrobial and antitubercular agents [13].
Now-a-days the world is facing an alarming situation because of lung cancer, which is the major cause of cancer death in man and women with the increasing mortality rate day by day [14]. According to data of 2012, 1.8 million People diagnosed with lung cancer and so far 1.6 million deaths reported due to this severe disease [15]. World health organization (WHO) report shows lung cancer five years survival rate (17.8%) is lower than another cancerous site such as Prostate (99%), breast (90.5%), colon (65.47%).

MATERIALS AND METHODS: Chemicals used in the synthesis of the title compounds described were purchased from S.D. Fine Chem. Ltd, Spectrochem Pvt. Ltd, Himedia and Loba Chemicals. They were different aromatic aldehydes, ethyl acetoacetate, piperidine, Mueller Hinton agar and Sabouraud Dextrose Agar. These chemicals were used as it is without further purification. All other LR grade reagents were used after purification using the literature methods. Melting points were determined with open capillary and are uncorrected. IR spectra were recorded in KBr pellets by using JASCO FT-IR 300E spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker - 400 MHz spectrometer using TMS as an internal standard.
Progress of the reaction and purity of the products were ascertained by thin-layer chromatography (TLC) using silica gel G as stationary phase and various solvent combinations as mobile phase; the spots were visualized by iodine vapors.

Preparation of 3-acetyl coumarin (I): To a mixture of salicylaldehyde (1.8gm, 0.02 M) and ethyl acetoacetate (2.5gm, 0.02 M), 2ml of piperidine was added by rapid stirring. After 20 minute the yellowish solid separated was filtered off and washed with ethanol. It was recrystallized from ethanol, it melts at 120°C (lit mp 120-122°C) and yield was 83.55%.

IR (KBr cm^{-1}): Characteristics peak at 1740.12 (lactone of coumarin); 1677.07 (Ketone C=O).

^{1}HNMR ($CDCl_3$): δ 2.73 (s, 3H, -CH_3); δ 7.32-7.68(m, 4H, -ArH) and δ 8.51 (s, 1H, C_4 of coumarin).

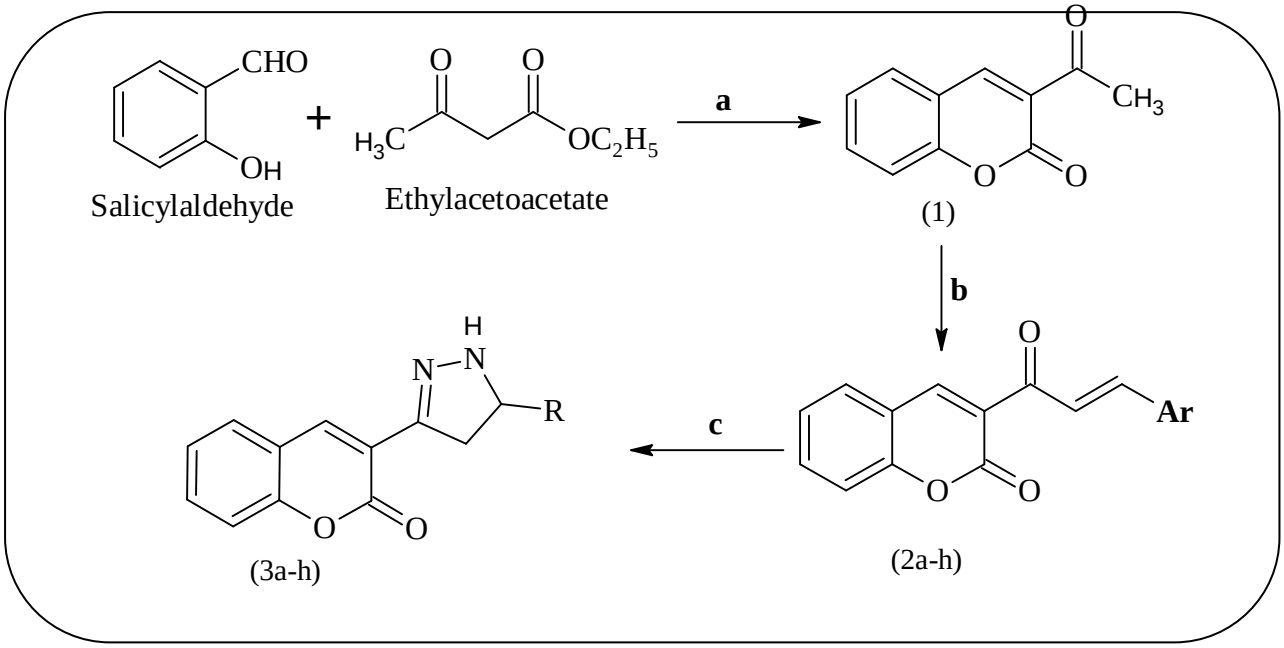
General procedure for the synthesis of 3-[(2E)-3-substituted-prop-2-enoyl]-2H-chromen-2-one (2a-h) [16]:

A mixture of 3-acetyl-2H-chromen-2-one (1) (1.88gm, 0.01M) and 0.012 M of the corresponding aromatic aldehydes was dissolved in 10 ml of n-Butanol under heating; then 0.3 ml of glacial acetic acid and the same quantity of piperidine were added. The reaction mixture was refluxed for 4 hr and then the solvent was removed under vacuum. The residue was triturated with 20 ml of ethanol until a precipitate formed, separated by filtration and recrystallized by suitable solvent. The physical data of the synthesized compounds **(2a-h)** are depicted in Table **1**.

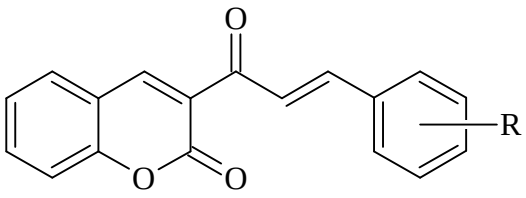
General procedure for the 3-[5-substitutedphenyl]-4, 5-dihydro-1H-pyrazol-3-yl]-2H-chromen-2-one (3a-h)

In a round bottomed flask a mixture of hydrazine hydrate 0.02 M and ethanolic solution (10 ml) of 0.01M chalcone was taken and refluxed for 3hr. The reaction mixture was poured on to crushed ice and stirred. The solid those obtained filter and wash with water and crystallized from appropriate solvents affording the corresponding (**3a-h)**.
The physico-chemical and spectral data of synthesized compounds **3a-h** is summarized in Table **2**.

Figure 1: Synthesis of 3-[5-substitutedphenyl]-4, 5-dihydro-1H-pyrazol-3-yl]-2H-chromen-2-one (3a-h)



*Reagents and conditions: (a) Piperidine, stirr, rt, 20 min; (b) Ar-CHO, Piperidine / n-Butanol, reflux, 4hr; (c) Hydrazine hydrate, ethanol.



(2a-h)

Table 1: Physico-chemical data of 3-[(2E)-3-substituted-prop-2-enoyl]-2H-chromen-2-one derivatives (2a-h).

Compound	R	Yield (%)	Solvent [a]	M. P. (^{0}C)	Rf [b]	Molecular Formula
2a	H-	90	Ethanol	170-172	0.88	$C_{18}H_{12}O_3$
2b	4-OMe-	43	Ethanol	154-156	0.57	$C_{19}H_{14}O_4$
2c	4-Cl-	46	Benzene	202-204	0.77	$C_{18}H_{11}O_3Cl$
2d	4-NMe_2-	72	Benzene	217-218	0.57	$C_{20}H_{17}NO3$

2e	3-NO_2-	85	Dioxane	225-228	0.65	$C_{18}H_{11}NO_5$
2f	4-Me-	75	Ethanol	170-172	0.66	$C_{19}H_{14}O_3$
2g	2-NO_2-	41	Ethanol	138-140	0.63	$C_{18}H_{11}NO_5$
2h	4-OH-	69	Dioxane	238-240	0.70	$C_{18}H_{12}O_4$

[a]Recrystlization solvent; [b]Chloroform:Water 7:3 as a mobile phase and iodine vapors as visualizing agent.

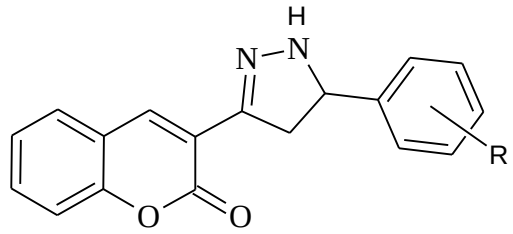


Table 2: Physico-chemical data of 3-[5-substitutedphenyl]-4, 5-dihydro-1H-pyrazol-3-yl]-2H-chromen-2-one (3a-h)

Compound	R[a]	Solvent [a]	Yield (%)	M. P. (^{0} C)	Rf [b]	Molecular Formula
3a	H-	Ethanol	69	175-180	0.38	$C_{18}H_{15}O_3N_2$
3b	4-OMe-	Ethanol	55	205-210	0.27	$C_{19}H_{16}O_3N_2$
3c	4-Cl-	Benzene	65	140-145	0.48	$C_{18}H_{13}O_2N_2Cl$
3d	4-NMe_2-	Benzene	62	135-140	0.46	$C_{20}H_{19}O_2N_3$
3e	3-NO_2-	Dioxane	65	105-110	0.25	$C_{18}H_{13}O_4N_3$
3f	4-Me-	Ethanol	74	110-115	0.36	$C_{19}H_{15}O_2N_2$
3g	2-NO_2-	Ethanol	70	120-125	0.33	$C_{18}H_{13}O_4N_3$
3h	4-OH-	Dioxane	67	215-220	0.42	$C_{18}H_{15}O_2N_2$

Rf[b] Chloroform: Methanol 10:1 as a mobile phase and iodine vapors as visualizing agent

3-[(2E)-3-Phenylprop-2-enoyl]-2H-chromen-2-one 2a:
IR (KBr cm^{-1}): 1731 (Lactone of coumarin), 1657(α, β-unsaturated ketone);
^{1}HNMR (CDCl3): δ 8.61 (s, 1H, 4th proton of coumarin), δ 7.83 (d, 1H, =CH-Ar), δ 7.43-7.78 (m, 9H, Ar-H), δ 7.41 (d, 1H, =CH-CO).

3-[(2E)-3-(4-Methoxyphenyl) prop-2-enoyl]-2H-chromen-2-one 2b:
IR (KBr cm^{-1}): 1732 (Lactone of coumarin), 1654 (α, β-unsaturated ketone);

^{1}HNMR (CDCl3): δ 8.58 (s, 1H, 4th proton of coumarin), δ 7.85 (d, 1H, =CH-Ar), δ 6.95-7.84 (m, 8H, Ar-H), δ 6.92 (d, 1H, =CH-CO), δ 3.86 (s, 3H, -OCH3).

3-[(2E)-3-(4-Chlorophenyl)prop-2-enoyl]-2H-chromen-2-one 2c:
IR (KBr cm^{-1}): 1716 (Lactone of coumarin), 1662 (α, β-unsaturated ketone);
^{1}HNMR (CDCl3): δ 8.60 (s, 1H, 4th proton of coumarin), δ 7.92 (d, 1H, =CH-Ar), δ 7.83 (d, 1H,=CH-CO), δ 7.34-7.69 (m, 8H, Ar-H).

3-{(2E)-3-[4-(Dimethylamino) phenyl] prop-2-enoyl}-2H-chromen-2-one 2d:
IR (KBr cm^{-1}): 1734 (Lactone of coumarin), 1657 (α, β-unsaturated ketone);
^{1}HNMR (CDCl3): δ 8.55 (s, 1H, 4th proton of coumarin), δ 7.85 (d, 1H, =CH-Ar), δ 7.75 (d, 1H,=CH-CO), δ 6.67-7.75 (m, 8H, Ar-H), δ 3.05 [s, 6H, -N(CH3)2].

3-[(2E)-3-(3-Nitrophenyl) prop-2-enoyl]-2H-chromen-2-one 2e:
IR (KBr cm^{-1}): 1712 (Lactone of coumarin), 1661(α, β-unsaturated ketone);
^{1}HNMR (CDCl3): δ 8.64 (s, 1H, 4th proton of coumarin), δ 8.49 (d, 1H, =CH-Ar), δ 8.27 (d, 1H,=CH-CO), δ 7.38-8.00 (m, 8H, Ar-H).

3-[(2E)-3-(4-Methylphenyl) prop-2-enoyl]-2H-chromen-2-one 2f:
IR (KBr cm^{-1}): 1731 (Lactone of coumarin), 1657 (α, β-unsaturated ketone);
^{1}HNMR (CDCl3): δ 8.57 (s, 1H, 4th proton of coumarin), δ 7.93 (d, 1H, =CH-Ar), δ 7.59 (d, 1H,=CH-CO), δ 7.21-7.87 (m, 8H, Ar-H), δ 2.39 (s,3H, -CH3).
3-[(2E)-3-(2-Nitrophenyl) prop-2-enoyl]-2H-chromen-2-one 2g:
IR (KBr cm^{-1}): 1712 (Lactone of coumarin), 1661 (α, β-unsaturated ketone);
^{1}HNMR (CDCl3): δ 8.64 (s, 1H, 4th proton of coumarin), δ 8.49 (d, 1H, =CH-Ar), δ 8.27 (d, 1H, =CH-CO), δ 7.38-8.00 (m, 8H, Ar-H).

3-[(2E)-3-(4-Hydroxyphenyl) prop-2-enoyl]-2H-chromen-2-one 2h:
IR (KBr cm^{-1}): 1729 (Lactone of coumarin), 1657 (α, β-unsaturated ketone);
^{1}HNMR (CDCl3): δ 9.63 (s, 1H, -OH), δ 8.55 (s, 1H, 4th proton of coumarin), δ 7.81 (d, 1H, =CH-Ar), δ 7.73 (d, 1H, =CH-CO), δ 6.89-7.72 (m, 8H, Ar-H).

3-(5-phenyl-4,5-dihydro-1H-pyrazol-3-yl)-2H-chromen-2-one 3a:
IR (KBr cm^{-1}): 3440 (NH), 2915 (CH2), 1596 (lactone of Coumarin), 1523 (C=C);
^{1}HNMR (CDCl3): δ 2.77 (dd, 1H, 4-Ht), δ 3.33 (dd, 1H, 4-Hc), δ 6.88 (dd, 1H, 5-H of pyrazoline), δ7.21 -7.84 (m, 9H, Ar-H), δ 7.65 (s, 1H, 4-H of coumarin), δ 9.02 (s, H, NH).

3-[5-(4-methoxyphenyl)-4, 5-dihydro - 1 H- pyrazol-3-yl]-2H-chromen-2-one 3b:
IR (KBr cm^{-1}): 3436 (NH), 2919 (CH2), 1592 (lactone of Coumarin), 1492 (C=C);
^{1}HNMR (CDCl3): δ 2.77 (dd, 1H, 4-Ht), δ 3.35 (dd, 1H, 4-Hc), δ 3.75 (s, 3-H, OCH3), δ 6.88 (dd, 1H,5-H of pyrazoline), δ 7.24 -7.84 (m, 8H, Ar-H), δ 7.65 (s, 1H, 4-H of coumarin), δ 9.02 (s, H, NH).

3-[5-(4-chlorophenyl)-4,5-dihydro-1H-pyrazol-3-yl]-2H-chromen-2-one 3c:
IR (KBr cm^{-1}): 3436 (NH), 2919 (CH2), 1677(lactone of Coumarin), 1565 (C=C);
^{1}HNMR (CDCl3): δ 2.78 (dd, 1H, 4-Ht), δ 3.34 (dd, 1H, 4-Hc), δ 6.88 (dd, 1H, 5-H of pyrazoline), δ7.32 - 7.84 (m, 8H, Ar-H), δ 7.65 (s, 1H, 4-H of coumarin), δ 9.02 (s, H, NH).

3-{5-[4-(dimethylamino)phenyl]-4,5-dihydro-1H-pyrazol-3-yl}-2H-chromen-2-one 3d:
IR (KBr cm^{-1}): 3350 (NH), 2965 (CH2), 1700 (lactone of Coumarin), 1550 (C=C);

^{1}HNMR (CDCl3): δ 2.76 (dd, 1H, 4-Ht), δ 3.02 (s, 6H, -NMe2), δ 3.35 (dd, 1H, 4-Hc), δ 6.54 (dd, 1H, 5-H of pyrazoline), δ 6.77-7.84 (m, 8H, Ar-H), δ 7.65 (s, 1H, 4-H of coumarin), δ 9.02 (s, H, NH).

3-[5-(3-nitrophenyl)-4, 5-dihydro-1H-pyrazol-3-yl]-2H-chromen-2-one 3e:
IR (KBr cm^{-1}): 3440 (NH), 2923 (CH2), 1697 (lactone of Coumarin), 1627 (C=C);
^{1}HNMR (CDCl3): δ 2.80 (dd, 1H, 4-Ht), 3.36 (dd, 1H, 4-Hc), 6.94 (dd, 1H, 5-H of pyrazoline), δ 7.31 – 8.394 (m, 8H, Ar-H), δ 8.40 (s, 1H, 4-H of coumarin), δ 9.02 (s, H, NH).

3-[5-(4-methylphenyl)-4, 5-dihydro - 1H-pyrazol-3-yl]-2H-chromen-2-one 3f :
IR (KBr cm^{-1}): 3436 (NH), 2919 (CH2), 1677 (lactone of Coumarin), 1573 (C=C);
^{1}HNMR (CDCl3): δ 2.21 (s, 3H, -CH3), δ 2.77 (dd,1H, 4-Ht), δ 3.33 (dd, 1H, 4-Hc), δ 6.88 (dd, 1H, 5-H of pyrazoline), δ 7.05 -7.84 (m, 8H, Ar-H), δ 7.65 (s, 1H, 4-H of coumarin), δ 9.02 (s, H, NH).

3-[5-(4-hydroxyphenyl)-4, 5-dihydro-1H-pyrazol-3-yl]-2H-chromen-2-one 3h:
^{1}HNMR (CDCl3): δ 2.77 (dd, 1H, 4-Ht), δ 3.34 (dd, 1H, 4-Hc), δ 6.73 (dd, 1H, 5-H of pyrazoline), δ7.16-7.84 (m, 8H, Ar-H), δ 7.65 (s, 1H, 4-H of coumarin), δ 9.02 (s, 2H, NH2), δ 9.18 (s, 1H, -OH).

Sulforhodamine B (SRB) Assay [17, 18]: The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37° C, 5 % CO2, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental drugs.
Experimental drugs were initially solubilized in dimethyl sulfoxide at 100mg/ml and diluted to 1mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquote of frozen concentrate (1mg/ml) was thawed and diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml with complete medium containing test article. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations i.e.10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml.
After compound addition, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on plate reader at a wavelength of 540 nm with 690 nm reference wavelength.
Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100.
Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was

calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as:
[Ti/C] x 100 %

Table 3: Human lung cancer cell line data of synthesized compound (3a-h)

Human Lung Cancer Cell Line A-549				
% Control Growth				
Drug Concentration (µg/ml)				
	Average Values			
Compound	**10**	**20**	**40**	**80**
3a	78.9	83.4	61.2	49
3b	100.3	96.9	84.5	73.5
3c	85.8	66.9	26.1	-0.4
3d	104.2	100.9	81.8	70.9
3e	97.1	93.9	76.8	65.4
3f	86.9	67.3	41.2	21.4
3g	108.6	95.3	65	18.9
3h	99.6	100.6	87.9	80.6
ADR	17.2	12.5	9.7	7.1

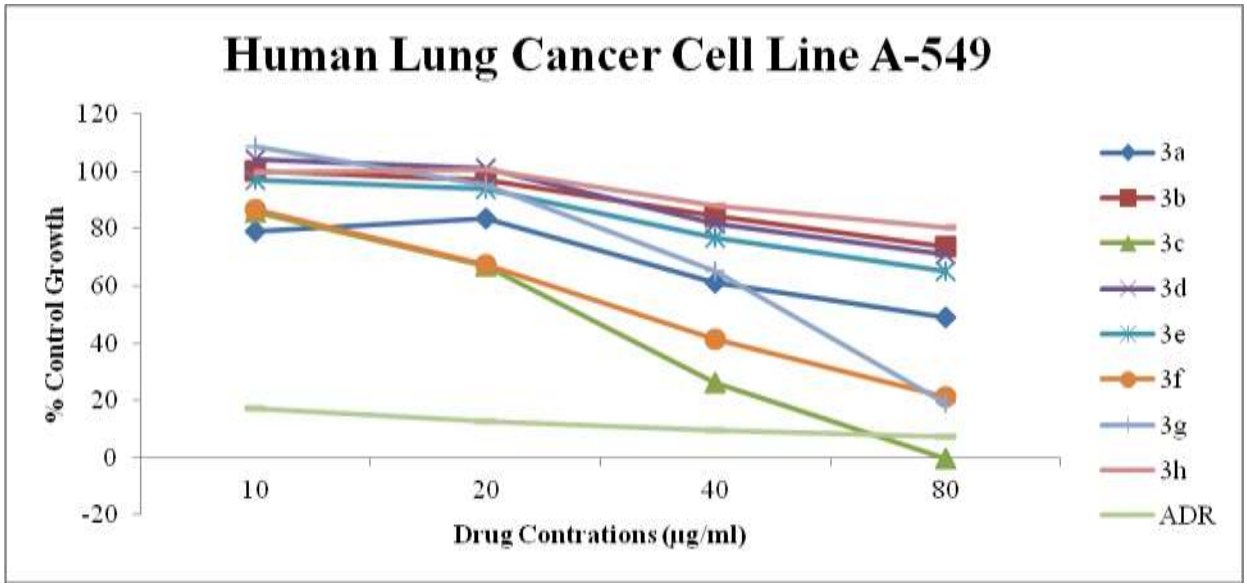


Figure 2: Sulforhodamine B assay in human lung cancer cell line A-549

RESULTS AND DISCUSSION:

3-Acetyl coumarin was synthesized by Knoevenagel condensation of salicylaldehyde with ethyl acetoacetate. Claisen-Schmidt condensation of 3-Acetyl coumarin with aromatic aldehydes gave 3-substituted cinnamoy lcoumarin. Treatment of 3-substituted cinnamoyl coumarin with hydrazine hydrate in ethanol gave 3-(5-substitutedphenyl)-4, 5-dihydro-1H-pyrazol-3-yl]-2H-chromen-2-one Fig. 1. The purity of the synthesized compounds was analyzed by thin-layer chromatography (TLC) on a silica gel G. The structure of synthesized compounds was characterized by spectral studies which include IR and 1H-NMR.

In IR spectra of compounds (2a-h) the characteristic peak of C=O of α-pyrone and C=O of ketone were observed at 1735.01 and 1656.10 cm^{-1} respectively; and in title compounds (3a-h) the characteristic peak of C=O of α-pyrone, C=N and C=C of pyrazoline were observed at 1720 -1730, 1585-1600 and 1533-1545 cm^{-1} respectively confirmed the structure of the title compounds. Further, the structure was ascertained by detailed 1H-NMR study of the compounds. In 1H-NMR spectra of compounds (3a-h), the presence of three doublet-doublet between δ 3.27 to 5.63 of $-CH_2$ and -CH of pyrazoline and multiplet between δ 6.85 to 8.42 is characteristic peaks of aromatic protons in spectrum of 3-[5-substitutedphenyl]-4,5-dihydro-1H-pyrazol-3-yl]-2H-chromen-2-one reveals confirmation of structures.

All these newly synthesized compounds (3a-h) were screened on A549 human lung cancer cell lines by Sulforhodamine B (SRB) assay. A morphological variation such as retardation in cell growth phenomenon was observed in A549 human lung cancer cell line treated with synthesized compound (Fig. 2).

CONCLUSION:

Among all the synthesized compounds some showed anticancer activity, further studies can be performed to evaluate the molecular mechanism behind anticancer activity along with their efficacy and safety profile.

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