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Synthesis, Characterization and Mechanistic Anticancer Evaluation of Novel Analogues of Pyrazoles Derived from Substituted 3-acetyl Coumarins

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In the present study, a congeneric series of novel substituted coumarin pyrazole carbaldehydes were synthesized. The compounds were characterized by various physical and spectroscopic methods. Preliminary cytotoxicity of the analogues was carried out using the MTT assay method on A-549 Lung cancer cell lines. The synthesized compounds possessed appreciable cytotoxicity against lung cancer cell lines. Out of the 8 synthesized compounds, the compound P-03 showed marked cytotoxicity of 13.5 mmol compared to standard doxorubicin which showed cytotoxicity value of 3.5 mmol. The compound P-03 was further investigated for its ability to induce apoptosis and its effect on cell cycle analysis. The compound P-03 was found to be an early apoptotic agent. After performing a cell cycle investigation, it was discovered that the compound P-03 effectively inhibited the G2/M phase of the cell cycle.

Keywords: Coumarin, Pyrazole, Hybrid, anticancer, mechanistic, cell cycle, apoptosis.

Various illnesses with the potential to infiltrate or spread to different body parts include cancer, which is a category of diseases characterized by abnormal cell proliferation. About 90-95% of cancers are due to genetic mutations, which transform normal cell into malignant cells.[1]Cancer cells acquire a degree of autonomy from mutations of tumor suppressor gene, resulting in uncontrolled cell growth and its proliferation.

According to the WHO study 2020, cancer cases found to be 18 million and 10 million mortalities worldwide in 2018 and by 2040, the global prevalence is projected to double, to 29-37 million new cancer cases[2]. It is also a big health concern that needs to be tackled. The development of new anticancer therapeutics is one of medicinal chemistry's top priorities because cancer accounts for roughly 70% of all fatalities. Because of the

high demand for anticancer drugs, medicinal chemistry researchers have focused their efforts on the chemistry and biology of new anticancer agents[3].

In recent years anticancer agents derived from natural products have gained the attention of researchers due to their wide range of therapeutic activity[4]. Coumarin, a versatile phenolic nucleus consisting of á -pyrone ring fused to a benzene ring that occurs naturally. Vogel first isolated it from tonka beans in 1820. Since tonka beans are rich in coumarins, the name came from the French word Coumarou. Coumarin-based compounds (Figure 1), both natural and synthetic, have anti-inflammatory, antibacterial, antiviral, antioxidant, and anticancer effects[5].

Along with coumarin, pyrazole analogs are found to possess anticancer antioxidant and anticancer activity[6]. In the last decade pyrazole gained much interest of researchers since the its structure is frequently found as active ingredient in commercial drugs.

Because of its wide variety of biological activities, Pyrazole and its derivatives have attracted a lot of interest in recent decades[7]. Molecular modeling is used by thousands of researchers to design novel pyrazole analogues that can target cancer-related receptors such as protein kinase, tyrosine kinase, and vascular endothelial growth factor (VEGF)[8].

Pyrazole, also called as 1,2 diazoles, contains three carbon atom and two nitrogen atoms present adjacent to each other which was denoted by the molecular formula $C_3H_4N_2$. The pKb value for pyrazole is 11.5 which explains the weak basic nature of the ring and pKa of conjugated acid of pyrazole was found to be 2.49 at 25°C. Ludwig Knorr invented the word pyrazole in 1883. The Pyrazole ring itself possesses therapeutic anticancer, analgesic, anti-inflammatory, antioxidant, antimicrobial, anticonvulsant, antiviral etc. activities (Figure-2)[9].

Ruxolitinib (blood cancer), Axitinib (renal cancer), Crizotinib (lung cancer), and other authorized anticancer medications all include the pyrazole ring as their fundamental core structure[10].

With this background, it was believed to synthesis some novel pyrazole analogues

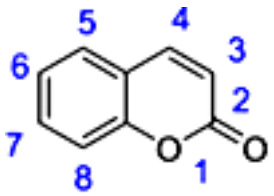


Fig. 1. Basic ring structure of Coumarin

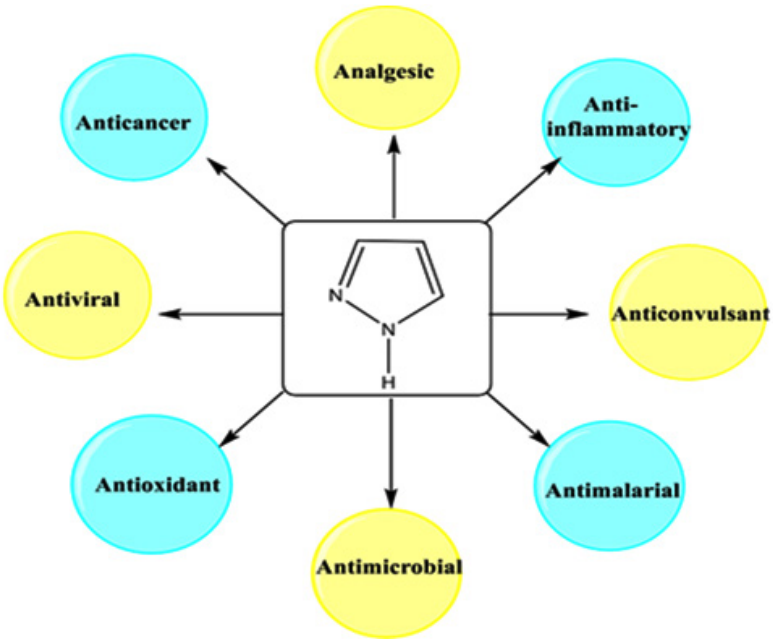


Fig. 2. Therapeutic properties of pyrazole nucleus

from substituted 3-Acetyl coumarin and test for antioxidant and antitumor activity.

MATERIAL AND METHODS

Chemicals and analytical instruments

All the chemicals and reagents used in this investigation were from TCI and Sigma Aldrich. Using CDCl3, DMSO as the solvent, tetramethyl silane as an internal standard, and a Bruker Avance II spectrophotometer, the spectra for ^{1}H NMR and ^{13}C NMR were obtained. The units used to report chemical changes were parts per million (ppm). Waters LCMS equipment were used to obtain all mass spectra. Melting points were also measured using an Electro Thermal 9100 tool without any post-processing.

The infrared spectra were captured using a Shimadzu spectrometer, and the absorptions were measured using a wave number (cm-1) scale that spanned from 400 to 4000 cm-1.The synthesis plan for the target compounds is shown in Figure 3.

General procedure

Step1 (Preparation of 3- acetyl coumarin)

Drop by drop, with constant stirring for five minutes, several salicylaldehyde derivatives each containing one equivalent (0.08 mole) of ethyl acetoacetate were added to the cool solution. About 5-6 drops of piperidine, the catalytic quantity, were added dropwise to the reaction mixture. Stirring continued for 6 to 48 hours. TLC was used to monitor the reaction's completion while utilising a 3:2 n-hexane:ethyl acetate solvent solution. The methanol-based solvent was extinguished when the reaction was finished, and the reaction mixture was then poured over crushed ice. Purification was achieved by recrystallizing the obtained precipitates in toluene after filtering them out to obtain the crude product.

Step-2 (Preparation of Coumarinyl hydrazones)

One equivalent of the 3-acetyl coumarin derivatives (Product I) was put to a round-bottomed flask and dissolved in 10 ml of glacial acetic acid. With constant stirring, one equivalent of methanol-

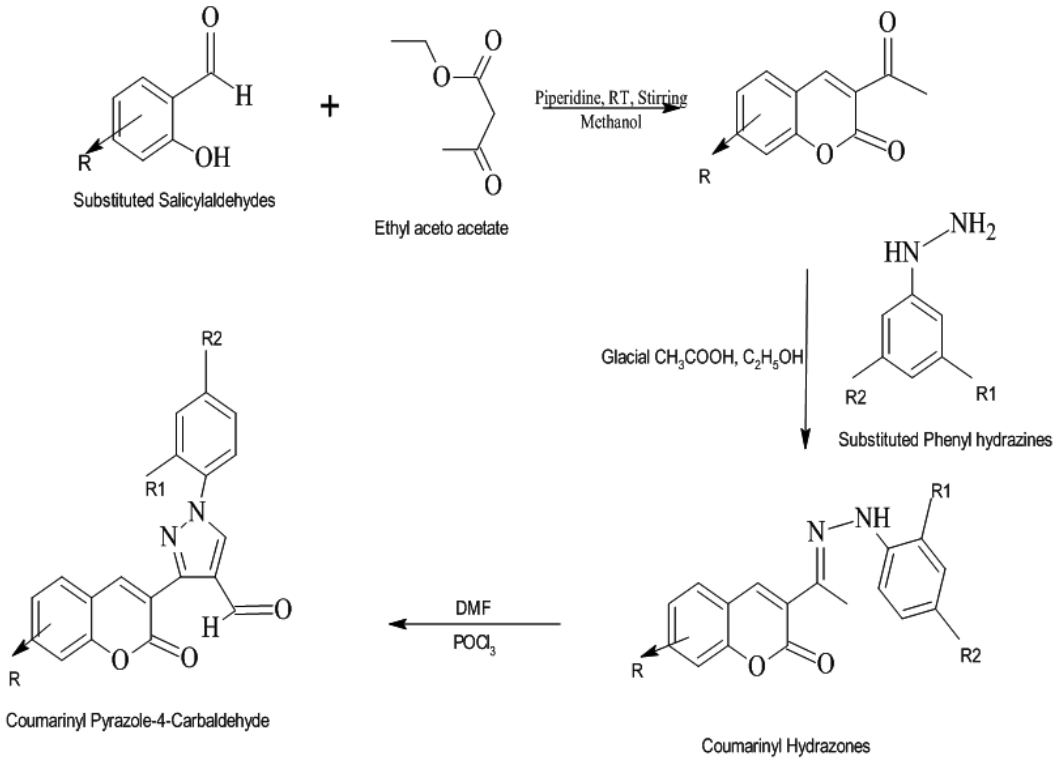


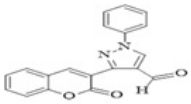
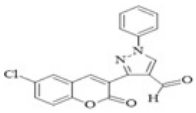
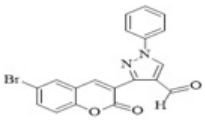
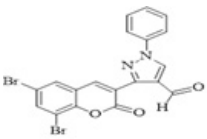
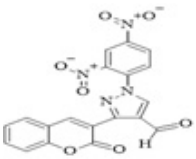
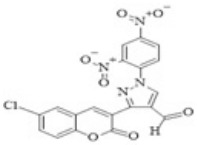
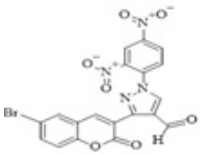
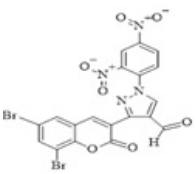
Fig. 3. General scheme for Coumarinyl pyrazole carbaldehyde synthesis

dissolved phenyl hydrazine derivatives was added to the solution. Using the solvent system n-hexane: ethyl acetate 3:2, stirring was continued for 1-2 hours until the reaction was complete. Orange-colored precipitates were seen after the reaction mixture was placed onto crushed ice. To achieve a pure product, the precipitates were filtered and washed with methanol.

Step-3 (Preparation of Coumarinyl pyrazole carbaldehyde's)[11]

POCl3 (5ml) was added dropwise while stirring to a 25ml cold solution of DMF to create

Table 1. Physico-chemical properties of synthesized test compounds

No	Code	Structure	MF	M.W.	M.P.	% Yield	Rf Value	Log P
1	P-01		$C_{19}H_{12}N_2O_3$	316.32	218-220	62.3	0.2	2.618
2	P-02		$C_{19}H_{11}C_lN_2O_3$	350.76	250-252	90.7	0.56	3.122
3	P-03		$C_{19}H_{11}BrN_2O_3$	395.21	258-260	93.81	0.56	3.497
4	P-04		$C_{19}H_{10}Br_2N_2O_3$	474.11	262-264	77.53	0.6	3.806
5	P-06		$C_{19}H_{10}N_4O_7$	406.31	222-224	66	0.36	1.310
6	P-07		$C_{19}H_9C_lN_4O_7$	440.75	216-218	78.06	0.4	1.816
7	P-08		$C_{19}H_9BrN_4O_7$	485.21	220-222	88.2	0.4	1.895
8	P-09		$C_{19}H_8Br_2N_4O_7$	564.10	208-210	76	0.3	2.51

the Vilsmeier reagent. 30 minutes later, 5 mmo1 of Coumarinyl pyrazoles were added, portion by portion, and stirred continuously for 24 hours. After the reaction was finished, the liquid was poured over crushed ice, where yellow solid precipitates were seen. The mixture was then neutralised with a strong solution of NaOH. Precipitates were obtained, filtered to yield crude product, and refined using column chromatography with a ratio of 7:3 n-hexane to ethyl acetate.

Characterization

The synthesized compounds were examined using physical and spectroscopic techniques. Spectroscopic characterization was performed using UV, IR, MASS and NMR spectroscopic methods[12]. UV spectroscopy helps in identifying conjugation in molecules as conjugation is directly linked to UV absorption. The infrared spectroscopy helps in identifying the various functional groups present in molecules with specific bending or stretching vibrations.

The mass spectrum identifies the molecular ion peak that further helps in identifying the molecular weight of any sample molecules. NMR spectrum identifies the various type of magnetically equivalent protons present in the given structure. Through the spectral assignment, one can deduce the structure of a given organic molecule.

Anticancer activity using MTT assay method using A-549(Lung cancer cell lines)

According to the established protocol described in the literature, the MTT assay was

Table 2. λ-max of synthesized compounds

Code	λ max (nm)
P-01	201.50
P-02	203.50
P-03	206.50
P-04	209.50
P-06	202
P-07	219
P-08	219
P-09	215

Table 3. IR functional group frequency values for the synthesized compounds

Functional groups	IR values (cm-1)
C=O	1724.36
Aldehydic C=O	1685.79
C=N	1608.63 and 2357.01
Aldehydic CH	2781.35 and 2856.58
C=C	1527.62
Aromatic CH	3055.24

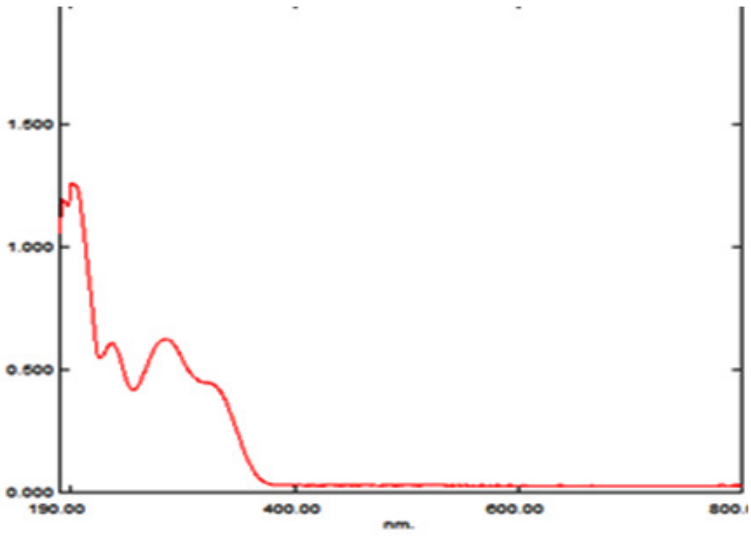


Fig. 4. UV Spectra of the compound P-01

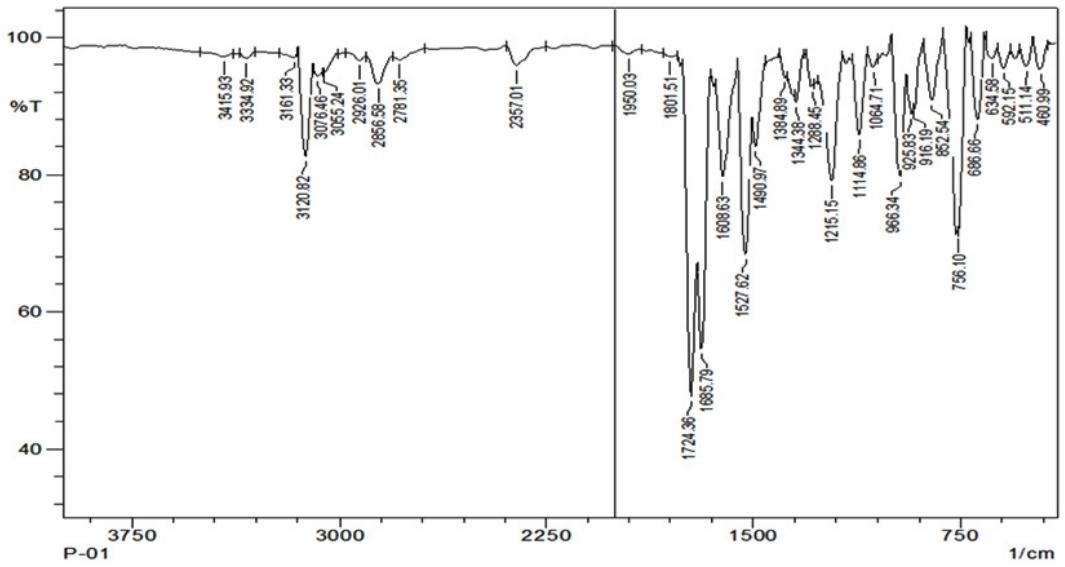


Fig. 5. IR spectra for the synthesized test compounds

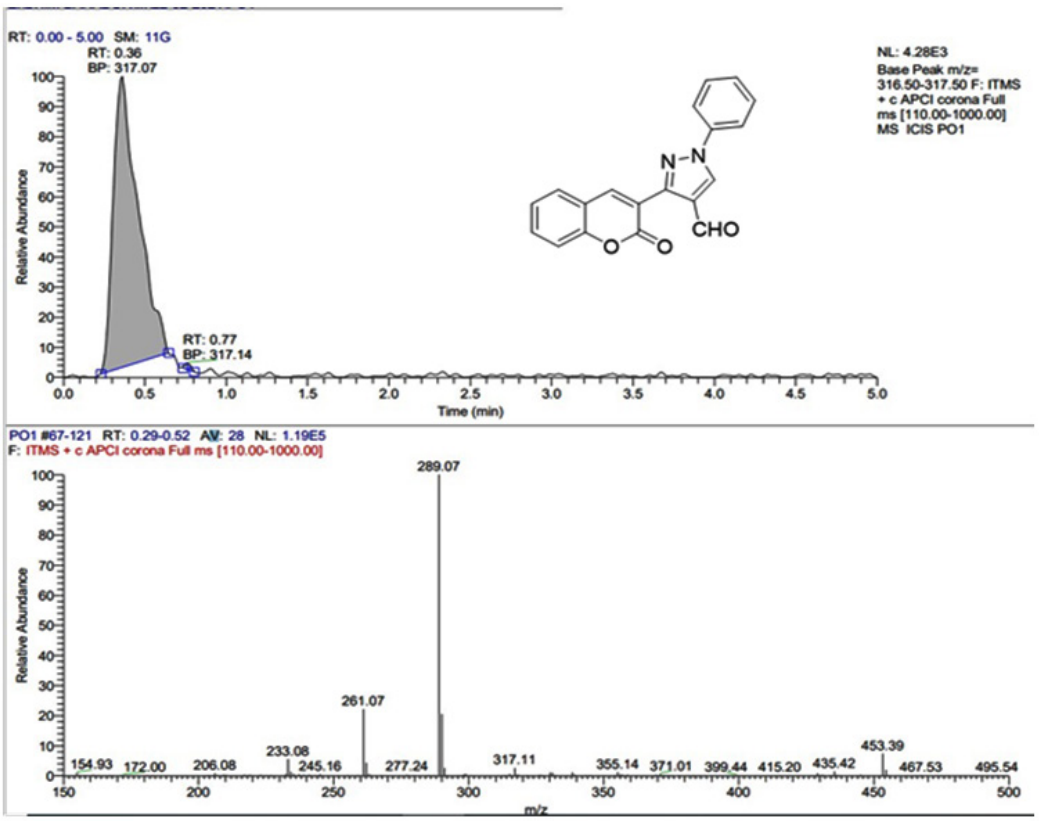


Fig. 6. Mass spectrum of the representative compound.

carried out[13]. The cells were seeded on the 96 well plates along with media and test solution. Cell cultures' media should be discarded. Aspirate the media slowly to check for adhering cells. For suspension cells, spin the 96-well plate at 1,000 x g at 4 °C for 5 minutes in a centrifuge that is compatible with microplates, then carefully aspirate the media. Each well should include 50 mL of serum-free medium and 50 mL of MTT solution. The plate should be incubated for three hours at 37 °C. After incubation, pour 150µL of MTT solvent into each well. The plate should be covered with foil and shook for 15 minutes using an orbital shaker. To completely dissolve the MTT formazan, the liquid might occasionally need to be pipetted. Read the absorbance at OD=590 nm.

PI Annexin V-FITC labelling for A549 cell apoptosis detection by flow cytometry.

According to the established protocol described in the literature, the apoptosis detection was carried out[14].

Stepwise procedure

1. On the day before apoptosis was induced, 1 X10^6 cells per well for a 6-well plate were seeded using medium containing 10% FBS and 1% Pen Strep, respectively. These cells were then incubated overnight at 37°C with 5% CO2.
2. Test solutions in medium containing 10% FBS were substituted for the original media.
3. Under standard culture conditions, the treated cells were incubated for 24 hours.
4. After the cells were removed from the wells, the entire contents were transferred to the sterile FACS tubes.
5. The supernatant from the centrifugation of the cell contents at 2000 rpm for 5 minutes was discarded.
6. After centrifugation, rinsed the cells twice with cold PBS before resuspending them in 1 mL of 1X Binding Buffer at a concentration of approximately 1 x 10^6 cells/mL.

Table 4. Cytotoxicity of the synthesized test compounds against A549 lung cancer cell lines.

Compound	IC_{50} mmol
P-03	13.5
P-04	25
P-01	18
P-09	31.3
P-07	37.7
P-02	29.23
P-06	32
P-08	21.67
Doxorubicin	3.63

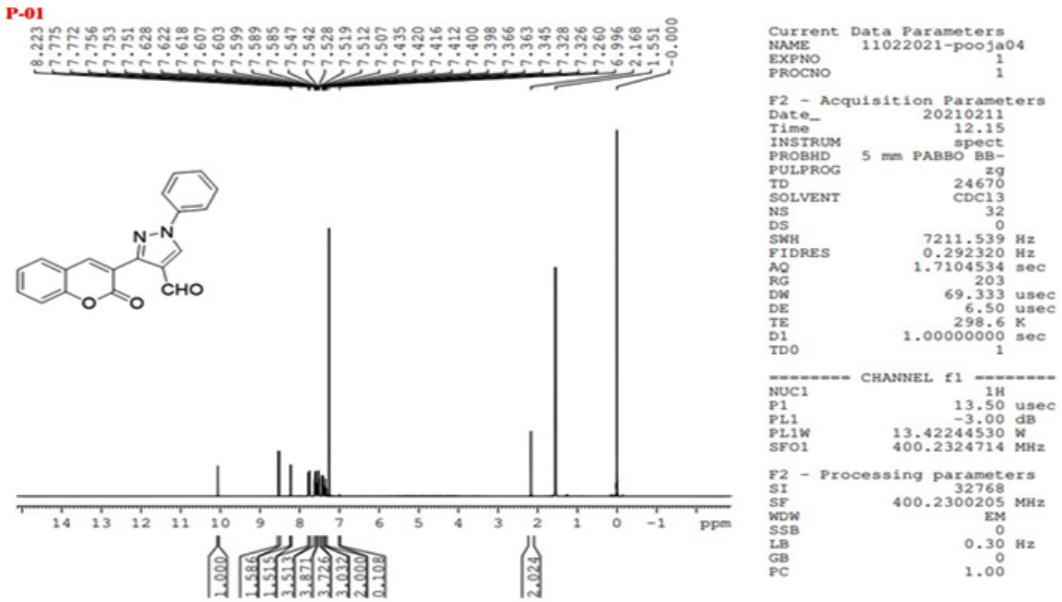


Fig. 7. ^{1}H NMR spectrum for the synthesized compound P-01

7. Transfer 500 mL (5 x 10^5 cells) of the cell suspension to a fresh FACS tube.
8. The tubes were filled with 8. 5 l Annexin V and 10 LPI, and the cells were then gently mixed before being incubated for 20 minutes at RT in the dark.
9. As quickly as feasible (within an hour), flow cytometry was used to analyze the cells.

Cell Cycle s

According to the established protocol described in the literature, the cell cycle analysis was carried out[15].

Procedure

In a 6-well plate with 2 ml of medium, 1 x 10^6 cells were planted and grown for 24 hours. Following that, cells were given the appropriate concentrations of the specified samples, prepared in medium, and cultured for an additional 24 hours. The cells were then collected, centrifuged for 5 minutes at room temperature at 2000 rpm, and the supernatant carefully discarded while still holding the cell pellet. After resuspending the cell pellet in 2mL of 1XPBS, it was cleaned. Another time, the washing was done under the same circumstances. The particulate was kept in the supernatant, which was discarded. After resuspending the cells in 300 l of Sheath fluid, 1 mL of cold 70% EtOH was added drop by drop while being continuously gently shaken, and a final 1 mL of chilled 70% EtOH was added slowly all at once. The cells were then kept at 4 °C either overnight or for 30 minutes. The cells

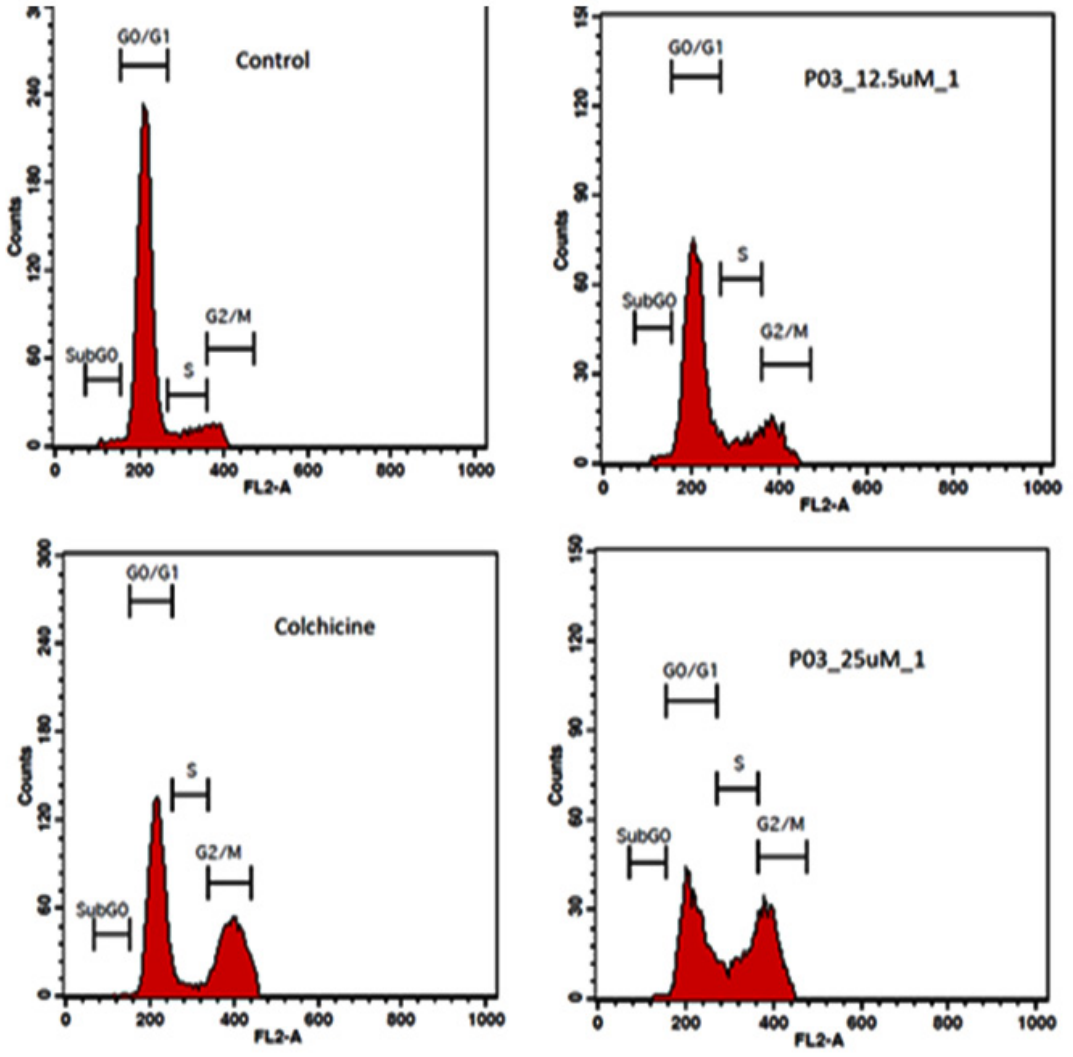


Fig. 7. Cell cycle analysis of Compound P-03 with control and standard doxorubicin

were centrifuged at 2000 rpm for 5 minutes after fixation. 2 ml of cold 1XPBS was used to wash the cell pellet twice. After that, the cell pellet was resuspended in 500 l of sheath fluid that included 0.05 mg/ml of PI and 0.05 mg/ml of RNaseA, and it was left to work for 20 minutes in the dark. Using FACS Calibre (BD Biosciences, San Jose, CA), it was possible to compare populations treated and untreated with drugs to assess the proportion of cells at different stages of the cell cycle.

RESULTS AND DISCUSSION

Synthesis

The target compounds were synthesized in 3 steps via formation of variety of intermediates.

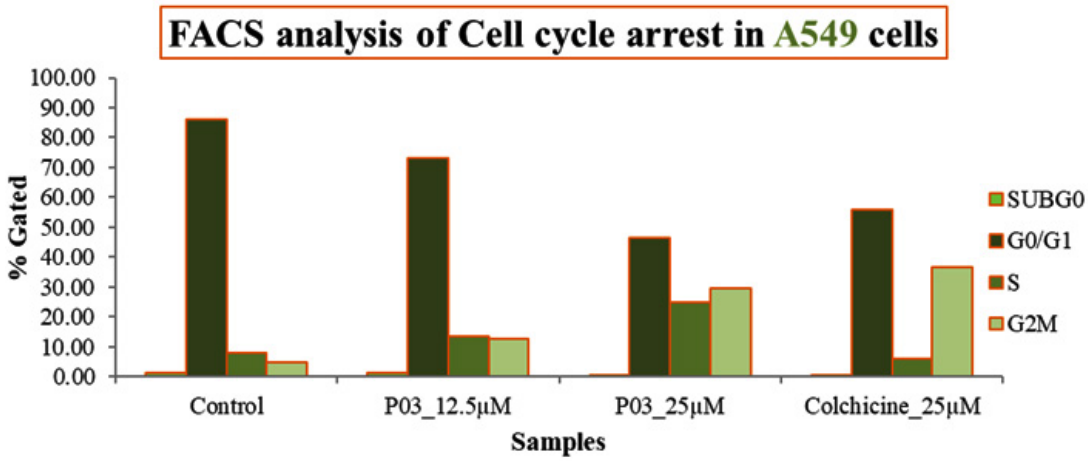


Fig. 8. Flow cytometry analysis of cell cycle arrest in A549 cells

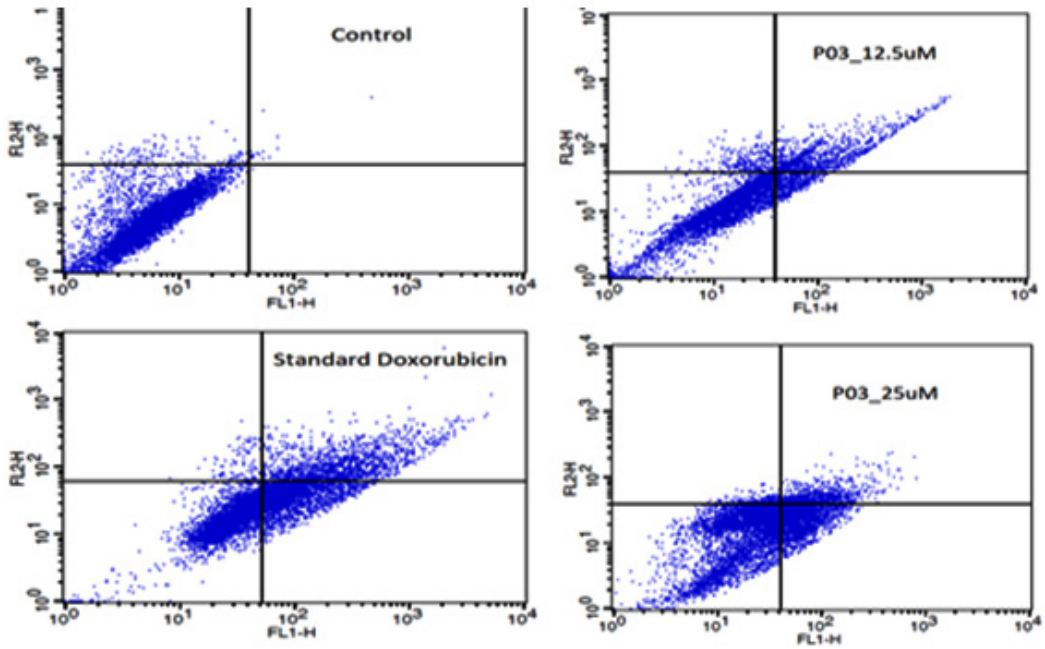


Fig. 9. Apoptosis detection with compound P-03 against A549 lung cancer cell lines with control and standard doxorubicin.

In the step 1, 3-acetyl coumarin was synthesized from the reaction of various substituted salicyl aldehydes and ethyl acetoacetate via Knoevenagel condensation. In the step 2, Coumarinyl hydrazones were synthesized by the nucleophilic addition of substituted phenyl hydrazine to 3-acetyl coumarin. In the final cyclization step, Vilsmeier Haack reaction was utilized for the preparation of various substituted Coumarinyl pyrazole derivatives. The structures and important physical properties for the synthesized compounds were presented in the table 1.

Characterization

The synthesized test compounds were characterized by various spectroscopic methods including UV, IR, MASS, and NMR spectroscopic methods[16].

In organic chemistry, the presence of free electrons or double (pi) bonds within a molecule can be determined using the UV/Visible spectroscopy approach. The term "Lamda-max" refers to the wavelength that a molecule absorbs most of, therefore it is possible to compare several compounds using this value[17].

The lambda max of all the 8 synthesized coumarin-pyrazole carbaldehydes were presented in Table 2.

The UV spectra of one of the representative compound P-01 was presented in the Figure 4.

IR spectroscopic analysis

IR spectra is useful to determine the functional groups present in the sample. Fingerprint region[16] of IR spectra is unique for each compound since different compounds have different natural frequencies of vibrations, no two organic compounds will produce a similar spectrum.

The IR spectra for the representative compound P-01 is given in Figure 4, the IR functional group values are provided in Table 3.

MASS Spectrometric analysis

The LC-MS technique makes use of HPLC to isolate individual components from the mixture, followed by ionization and according to mass/charge ration ions are separated, directed into detector which recognizes and quantifies each ion[18,19].

The popular Atmospheric Pressure Chemical ionization is the ion source used in LC-MS to generate ions from intact molecules. Since the LC-MS technique is precise, specific, sensitive the analysis is made at molecular level, it is easy to figure out structural details of the injected analyte.

The LCMS spectra for the representative compound P-01 is represented in Figure 5.

LC-MS (ITMS + cAPCI corona Full ms): Calculated for C19H12N2O3 [M+H]+ 317.32, found 317.11

Table 5. Flow cytometry analysis of cell cycle arrest in A549 cell

	FACS analysis of Cell cycle arrest in A549 cells			
Samples	SUBG0	G0/G1	S	G2M
Control	1.30	86.08	8.10	4.88
P03_12.5µM	1.32	73.14	13.48	12.74
P03_25µM	0.44	46.38	24.64	29.42
Colchicine_25µM	0.12	55.96	5.85	36.55

Table 6. Flow cytometry analysis of Apoptosis detection in A549 cell lines

	FACS analysis of Apoptosis detection in A549 cells			
Sample	Viable cells	Early Apoptotic	Late Apoptotic	Necrotic cells
Control	98.58	0.01	0.17	1.24
P03_12.5µM	77.14	5.89	13.42	3.55
P03_25µM	55.42	29.82	11.22	3.54
Doxorubicin_25µM	51.98	31.97	14.96	1.10

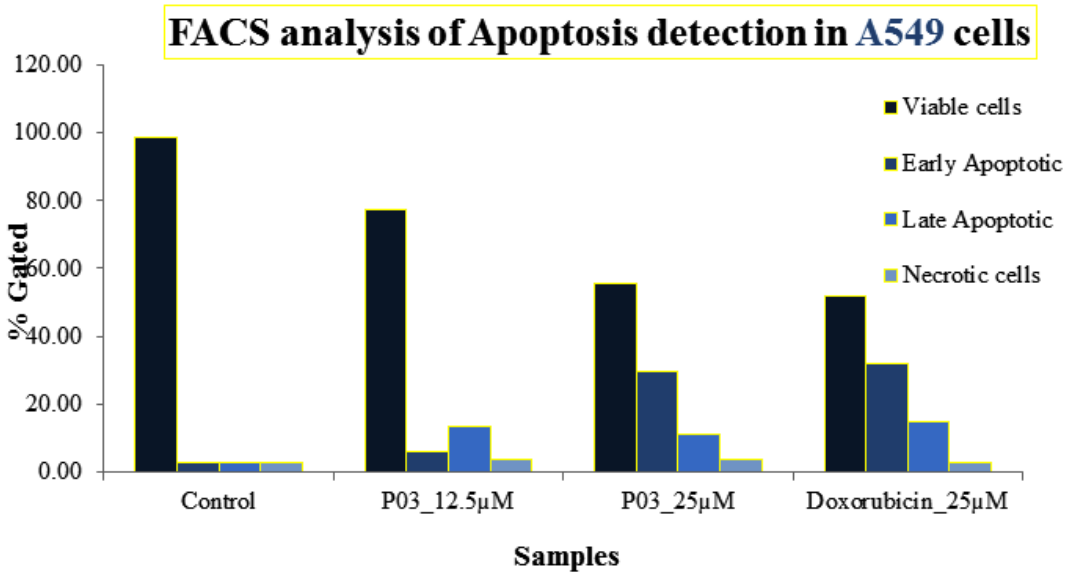


Fig. 10. Flow cytometry analysis of Apoptosis detection in A549 cell lines

NMR spectroscopic analysis

NMR helps us by providing the information about different magnetically distinct atoms of the provided be it hydrogen or carbon. Also, it gives brief idea about the nature of immediate environment of each proton. So, it helps in determining the structure of the compound.

In the ^{1}H NMR of the representative compound P-01 given below the characteristic peaks[20]. corresponding to protons were as follows

^{1}H NMR (400MHz, CDCl3, ä in ppm): 10.061(1H, s, CHO), 8.525(1H, s, CH-pyrazole), 8.223(1H, s, CH of pyrone ring), 7.26-7.77 (9H, m, Ar-H)

The ^{1}H NMR spectrum for the representative compound P-01 is presented in Figure 6.

Anticancer activity

The cytotoxicity studies of the synthesized test compounds were performed on A549 lung cancer cell lines. All the synthesized compounds possessed appreciable anticancer activity against lung cancer cell lines. Out of the 8 compounds tested, the compound P-03 showed the most prominent anticancer activity with an IC_{50} of 13.5 mmol in comparison to the standard doxorubicin that showed an IC_{50} value of 3.63 mmol. Results of anticancer studies were presented in Table 4.

To further substantiate their potential as anticancer agents, mechanistic studies including cell cycle analysis and apoptosis studies were performed.

Cell cycle analysis

The treatment of A549 cells at the concentrations of 12.5µM and 25µM with sample *P03* has shown S phase and G_2M phase arrest of 13.48%, 24.64% and 12.74%, 29.42% respectively.

Standard *Colchicine* at 25µM showed a G_2M arrest of 36.55% in A549 cells as shown in the Figure 7.

The cell cycle analysis suggested the potential of the synthesized compound to inhibit the cell cycle at G2/M phase. The cell cycle analysis data was presented in Table 5 and Figure 8.

Apoptosis detection

The sample *P03* treated at 12.5µM and 25µM has induced 5.89%, 29.82% early apoptosis and 13.42%, 11.22% late apoptosis in A549 respectively. Standard *Doxorubicin* at 25µM has shown total apoptosis of 46.93% in A549 cells as shown in the Figure 9.

The apoptosis studies suggested the potential of the synthesized compound in inducing late apoptosis. The FACS apoptosis data was presented in Table 6 and Figure 10.

CONCLUSION

A series of novel substituted coumarin pyrazole hybrids were synthesized utilizing the concept of hybridization as a lead optimization approach. The synthesized compounds were characterized by physical as well as spectroscopic techniques. Anti cancer activity of the compunds was performed on A-549 lung cancer cell lines against standard doxorubicin. Out of the 8 compounds synthesized, the compound P-03 emerged as a potent antiproliferative agent with an IC50 value of 13.5 mmol in comparison to standard doxorubicin that possessed an IC50 value of 3.63 mmol. The presence of bromine attached to the coumarin moeity significantly enhanced its anticancer potential. To substantiate the results, mechanistic anticancer studies including cell cycle analysis and apoptosis detection studies were performed. Cell cycle analysis further confirmed its ability to inhibit cell cycle at G2/M phase. Apoptosis studies confirmed its role as a late apoptotic agent.

In future studies, an attempt will be made to study their anticancer potential in animal models to reconfirm its ability against lung cancer models.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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