MS.ID-IJPBS-2561
International Journal of Pharmacy and Biological Sciences-IJPBS™ (2024) 14 (2): 73-82
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Pharmaceutical Sciences | OA Journal | MCI Approved | Index Copernicus

In-Silico Design, Synthesis and Evaluation of Some Pyrazolo Pyrazole Derivatives as Anticancer Agents

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Received: 18 Jan 2024 / Accepted: 16 March 2024/ Published online: 01 April 2024
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Abstract

Pyrazole is chemically known as 1,2-diazole and has become a popular topic of study. The chemistry of pyrazoles and its derivatives is particularly interesting because of their potential application in medicinal chemistry as analgesics, anti-inflammatory, antibacterial, antimalarial, antifungal, and as enzyme inhibitory agents The discovery of new drugs require not only its design and synthesis but also the development of testing method and procedures, which are needed to establish, how a substance operates in the body and its suitability for use as a drug. The aim of the study is to design different pyrazole derivatives using various softwares (eg; ACD lab Chemsketch 12.0, Molinspiration, PASS, Schrodinger). The designed compounds were synthesized. Characterization of synthesized derivatives was done by 1H NMR, MASS and IR spectroscopy. Synthesized derivatives were screened for anticancer activity.

Keywords

ADME: Adsorption Distribution Metabolism and Excretion **cm**: Centi meter, **FTIR**: fourier Transform Infrared Spectroscopy, **GCMS**: Gas chromatography coupled with mass spectroscopy, **GLIDE**: Grid based ligand docking with energies, **Hrs**: Hours, **KBr**: Potassium bromide, **mg**: Milli gram, **MTT**: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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INTRODUCTION:

Drugs are chemicals that prevent diseases or assist in restoring health to diseased individuals. As such they play an indispensable role in modern medicines. Drugs may be mere chemicals, but they are entering a word of chemical reactions with which they interact. Drug design, sometimes referred to as rational drug design, is an inventive process of finding new medications based on the knowledge of biological target. Drug design involves the design of molecules that are complementary in shape and charge to the biomolecular target with which they interact and therefore will bind to it. [1]

The incorporation of heterocyclic ring into prospective pharmaceutical candidates is a major tactic to gain activity and safety advantages. Pyrazoles are known not only as potent insecticides, herbicides, and monomers for the preparation electroluminescent and thermo resistant materials, but also as antitumor, anti-inflammatory,

antimicrobial, antipsychotic or analgesic agents. Thus, due to their wide range of pharmacological and technological applications, pyrazoles have been the focus of much synthetic effort in the past decades. [2] Cancer still remains one of the most feared disease in the modern world. Cancer is a class of disease in which a group of cells display uncontrolled growth, invasion and sometimes metastasis. These three properties differentiate cancer. Most cancers can be treated and some cured, depending on the specific type, location and stage. Once diagnosed, cancer is usually treated with a combination of surgery, chemotherapy and radiotherapy. Here in this article a series of pyrazole analogues designed, synthesized and evaluated its anticancer activity. [3]

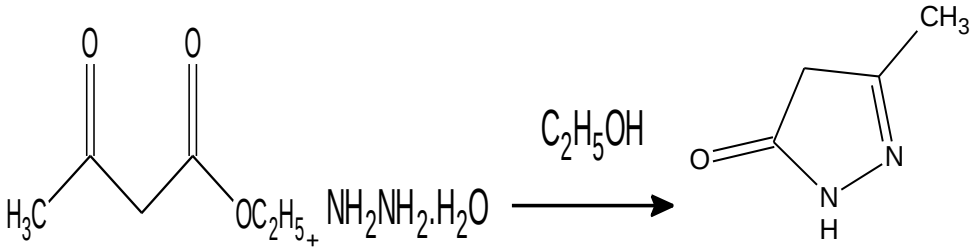
MATERIALS AND METHODS:

All the chemicals and reagents used were analytical grades. Softwares such as Molinspiration, Chemsketch, PASS, were used for the *in-silico* screening of the molecules and to calculate their physicochemical properties, drug likeness. Docking studies were carried out using Schrodinger. Conventional method of synthesis: There are 3 steps in the synthetic part. First step is the reaction between ethyl acetoacetate and hydrazine hydrate in presence of ethanol to form 3- methyl 5-pyrazolone which is a cyclization reaction. In 2nd step it undergoes react with different aldehydes in presence of acetic acid again on treating with hydrazine hydrate/ thiosemicarbazide to form pyrazolopyrazole derivatives. [3, 4] The synthesized compounds were purified by recrystallization. The individual products synthesized in each step were purified by recrystallization using appropriate solvents. Here ethanol is used as solvents. Characterization of the compounds can be done routinely by thin layer chromatography[2]. It also used to determine the completion of the reaction. Silica gel used as stationary phase. And the mobile phase is benzene, chloroform, and glacial acetic acid in the ratio of 2: 4: 1 and ethyl acetate, petroleum ether in the ratio of 7:3 Iodine chamber is used to detect the spot. [5] Melting point is determined by digital melting point apparatus. IR spectra of the compound were recorded using KBr pellets in the range of 4000-500 cm^{-1} on FTIR model 6200 in the Department of Pharmaceutical Analysis, College of Pharmaceutical sciences, Medical College, Trivandrum. Mass spectra of the compounds (GCMS) were recorded by at Department of applied chemistry, CUSAT, Cochi. NMR spectra of the compounds were recorded on Bruker Avance AV 500 spectrometer at 500 MHz, at Indian Institute of Science Education and Research (IISER), Thiruvananthapuram.

SYNTHETIC PROCEDURE

All reactions were performed using reagents and solvents of analytical grade.

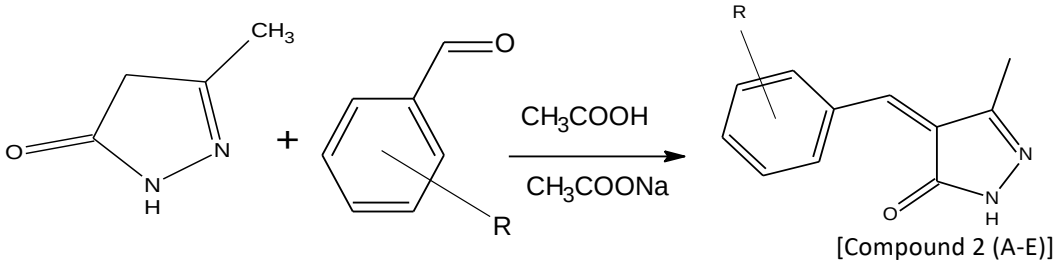
STEP 1 (synthesis of 3-methyl 5-pyrazolone)



Ethyl acetoacetate **Hydrazine hydrate** **3-Methyl 5-pyrazolone**

65 g (0.5 mole) ethyl acetoacetate was taken in a 250 ml conical flask and stirred magnetically. Added of a solution of 25 g (0.5 mole) of hydrazine hydrate in 40 ml of absolute ethanol to the conical flask. The temperature of the reaction mixture increased during reaction so that temperature was regulated at 60°C. A crystalline deposit was separated after stirring for 1 hour at 60°C. The reaction mixture was cooled in an ice bath to complete the crystallization. After standing for some time for completion of crystallization, filtered and washed with cold alcohol and recrystallized from alcohol. [6, 7]

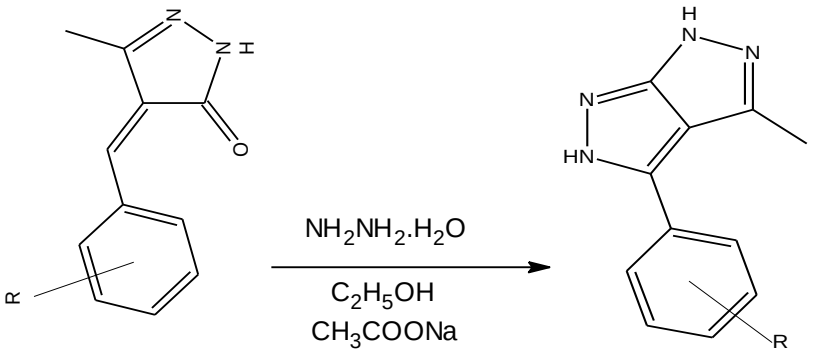
STEP II (synthesis of (4E) - substituted 4-benzylidine-5-methyl-2,4-dihydro-3H-pyrazol-3-one)



[Compound 2 (A-E)]

3-methyl 5-pyrazolone (0.01 mole) and aromatic aldehyde (0.01 mole) were refluxed in acetic acid in presence of anhydrous sodium acetate (0.01 mole) for 8 hours. The reaction mixture was cooled to room temperature and poured in ice cold water. The solid separated out was filtered washed with water and recrystallized from ethanol. [8,9]

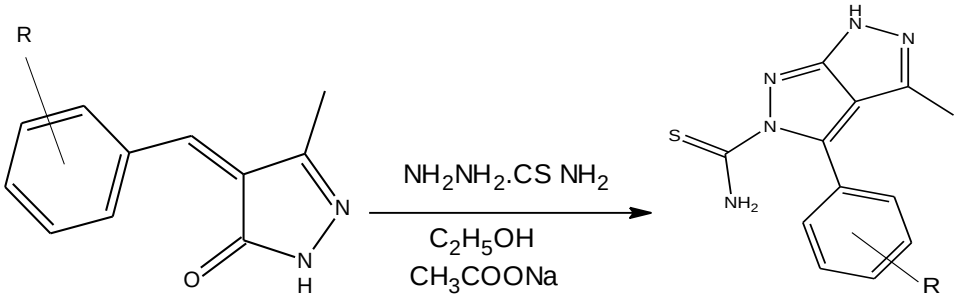
STEP III (a) (synthesis of 3-methyl substituted 4-phenyl -1,5-dihydropyrazolo [3,4-c]pyrazol)



[Compound PYR (1, 3, 5, 7, 9)]

Compound 2 (0.01 mole) and hydrazine hydrate (0.01 mole) refluxed in ethanol in presence of anhydrous sodium acetate (0.01 mole) for 10 hrs. Reaction mixture was cooled to room temperature and poured in ice cold water. The solid separated out was filtered, washed with water and recrystallized from ethanol. [10,11,12]

STEP III (b) Synthesis of Substituted 3-phenyl pyrazolo[3,4-*c*] pyrazole-2(6*H*)-carbothioamide



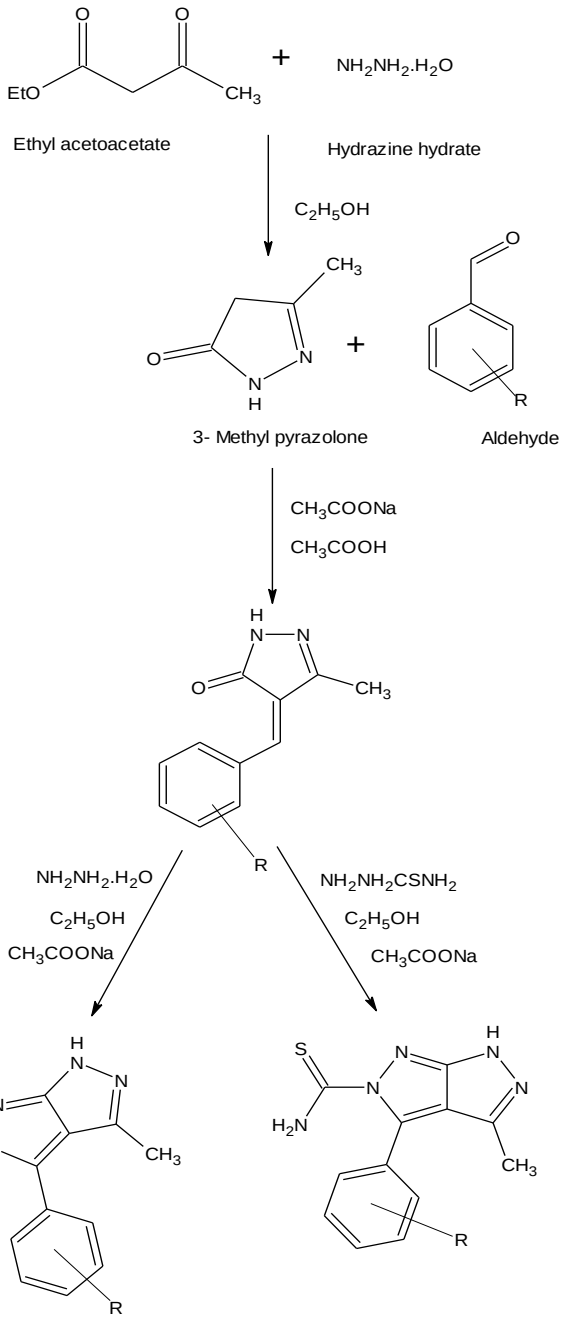
[Compound PYR (2, 4, 6, 8, 10)]

Mixture of compound 2(0.5mol) and thiosemicarbazide (0.5 mol) refluxed in ethanol in presence of anhydrous sodium acetate (0.5 mol) for 6 hours. Reaction mixture was cooled to room temperature and poured in ice cold water. The solid

separated out was filtered, washed with water and recrystallized from ethanol. [13,14,15]

This general procedure was adapted for the synthesis of all derivatives

SCHEME OF WORK



PHARMACOLOGICAL SCREENING

Anticancer studies

The cytotoxic screenings of the compounds were done by using the MTT assay method. The screening was carried out at college of pharmaceutical sciences, Govt. Medical College, Trivandrum

Cell lines used: Human colon cancer cell lines (HCT 116)

Stain used: MTT

Standard: 5-f; urouracil

Control: DMSO

Reagent preparation

Dissolve the MTT powder 5mg/ml in phosphate buffer saline solution. (eg; 100mg MTT/ 20ml PBS). When not in use protected the reconstituted MTT regent from light and stored at 4°C. Seeded cells at

3×10^5 cells/well in a 96 well plale. Left atleast 3 wells without cells. These wells as a control for the minimum absorbance. Incubate the plate overnight at 37°C in a humidified incubator, 5% CO_2. Added test compounds to the plate. Replicate for a range concentrations. Include negative control and a positive control. The final volume was 100µl per well. Incubated the plate 48 hours at 37°C in a humidified incubator, 5% CO_2. Added MTT reagent (10µl/100 µl per well of the 96 well plate). Covered the plate with aluminium foil and incubated at 37°C for 4 hours. [16,17,18,19]

After incubation the spent media is removed without disturbing the formazan crystals. 100 µl of isopropyl alcohol was added and kept for 15 minutes in a shaker so that formazan crystals get dissolved. Read the plate on a plate reader using 570 nm as test wavelength and 630 nm as the reference wavelength. Record the data in an excel spreadsheet, saved with a unique identifier. Tabulate result and calculated the % viability:

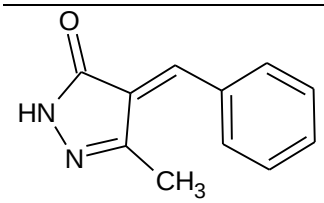
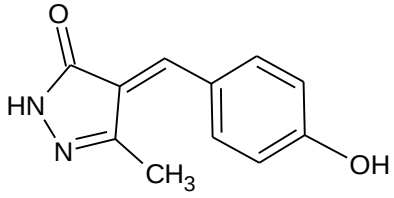
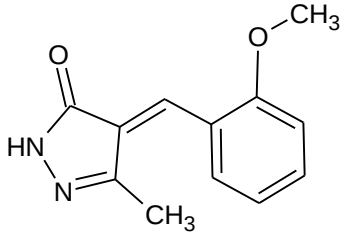
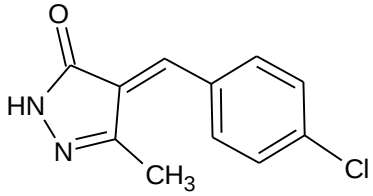
$$\% \text{ viability} = \frac{\text{Mean Absorbance of sample} \times 100}{\text{Mean Absorbance of control}}$$

The area of the well is approximately 0.27cm. A vehicle control is maintained for each different solvent used to dissolve test compound. [20,21]

RESULTS AND DISCUSSIONS:

In-silico design was successfully carried out with the aid of commercially available softwares (Molinspiration, ACD lab Chemsketch 12.0, PASS and Schrodinger) for the selection of suitable drug candidates prior to the wet lab synthesis. Lipinski rule of five and drug likeness profile were carried out using Molinspiration software. Molecular descriptors were determined by ACD Lab Chemsketch. Biological activity of selected pyrazole derivatives were predicted by PASS software. All the proposed derivatives were subjected to docking using GLIDE XP (Extra precision). Using Qikprop, an ADME prediction program provided by Schrodinger under Mastero, all the proposed analogues were efficiently evaluated for pharmaceutically relevant properties within limited time fractions, making an indispensable lead generation and optimization tool. Out of 16 designed derivatives, 1o derivatives were synthesized by wet lab synthesis. [22,23]

Table 1: Characterization of step II derivatives

Structure	Compound code	Mol formula	Percentage yield(%)	Melting point(°C)	Rf value
	Compound 2A	$C_{11}H_{10}N_2O$	63	232	0.69
	Compound 2B	$C_{11}H_{10}N_2O_2$	67	233	0.66
	Compound 2C	$C_{12}H_{12}N_2O_2$	62	236	0.68
	Compound 2D	$C_{11}H_9N_2OCl$	66	235	0.69

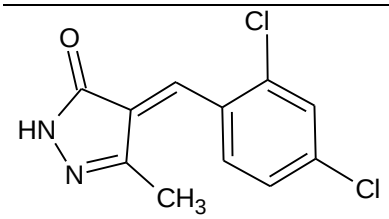
	Compound 2E	$C_{11}H_8N_2O_2$	61	237	0.67

Table 2: Characterization of pyrazole derivatives

Compound Code	Mol.formula	Mol.wt	Yield (%)	m.p (°C)	Rf
PYR 1	$C_{11}H_{10}N_4$	198.23	54	213	0.64
PYR 2	$C_{12}H_{11}N_2S$	257.32	55	214	0.58
PYR 3	$C_{11}H_{10}N_4O$	214.23	57	217	0.57
PYR 4	$C_{12}H_{11}N_5OS$	273.32	58	209	0.59
PYR 5	$C_{12}H_{12}N_4O$	228.25	50	210	0.51
PYR 6	$C_{13}H_{13}N_5OS$	287.35	61	221	0.56
PYR 7	$C_{11}H_9ClN_4$	232.67	58	220	0.59
PYR 8	$C_{12}H_{10}ClN_5S$	291.77	63	211	0.55
PYR 9	$C_{11}H_{10}N_4O$	214.23	56	212	0.63
PYR 10	$C_{12}H_{11}N_5OS$	273.32	60	207	0.61

ANTICANCER – **TYROSINE PROTEIN KINASE ENZYME (3KKR)**

Table 3: Glide score of derivatives with 3KKR

Sl.No	Compound code	Glide Score
1	PYR 1	**-7.726**
2	PYR 2	-5.734
3	PYR 3	-7.504
4	PYR 4	**-8.387**
5	PYR 5	-7.026
6	PYR 6	-8.802
7	PYR 7	-8.045
8	PYR 8	-8.354
9	PYR 9	-8.858
10	PYR 10	-8.424

Fig 1: 3D image of PYR 6 with 3KKR

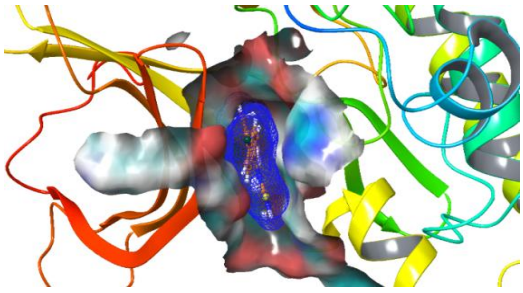


Fig 2: 2D image of PYR 6 with 3KKR

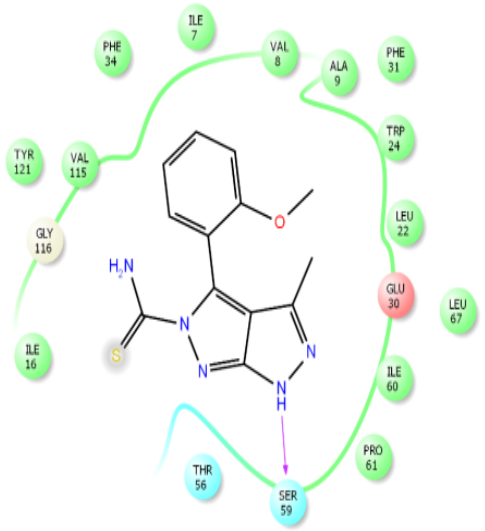


Table 4: Interactions involved in docking with 3KKR

Compound code	Pyrazole nucleus	Aromatic aldehyde	Hydrogen bonding
PYR 4	VAL 911, ALA 880, hydrophobic	ASP 994, ARG 980, charged. ASN 981, polar	NH of the pyrazole with GLU 930
PYR 6	VAL 911, ALA 880, MET 929, hydrophobic. LYS 882, charged	VAL 563, LEU 858, hydrophobic. ARG 980, charged. ASN 981, polar	NH of the pyrazole with GLU 930

In vitro anticancer activity of the synthesized pyrazole derivatives were done by MTT assay method against HCT 116 (Colon cancer cell line). PYR 2, PYR 4 and PYR 6 were opted for antiproliferative activity on the basis of their glide score. And the results were compared with 5-Flurouracil. [24,25]

Table 5, 6: anticancer activity of selected pyrazole derivatives on Colon cancer cell lines (HCT 116)

Concentration (µg/ml)	PYR 2		PYR 4	
	Absorbance	% viability	Absorbance	% viability
10	0.813	82.37	0.742	75.15
20	0.710	71.93	0.613	62.10
30	0.521	52.7	0.409	41.84

Concentration (µg/ml)	PYR 6		5-flurouracil	
	Absorbance	% viability	Absorbance	% viability
10	0.792	80.24	0.548	55.5
20	0.723	73.25	0.495	49.15
30	0.509	51.57	0.301	30.39

DISCUSSION:

MTT assay of PYR deivatives on HCT 116 cell lines showed that the activity of proposed analogues is less than that of standard 5-flurouracil. [26] The compound PYR 4 showed more anticancer activity than PYR 2 and PYR 6. The order of activity was found to be PYR 4 > PYR 6 > PYR 2.

Fig 3: percentage viability of PYR derivatives.

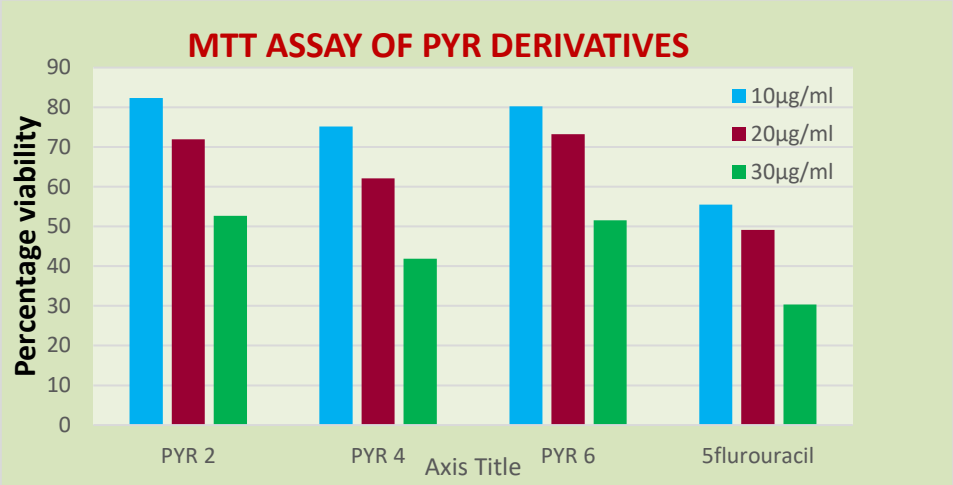
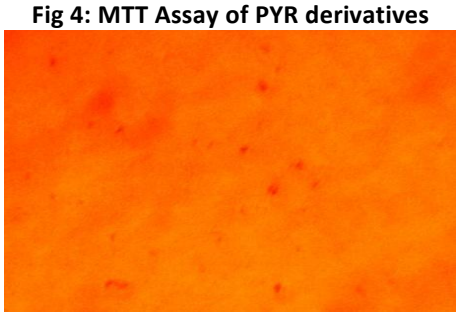
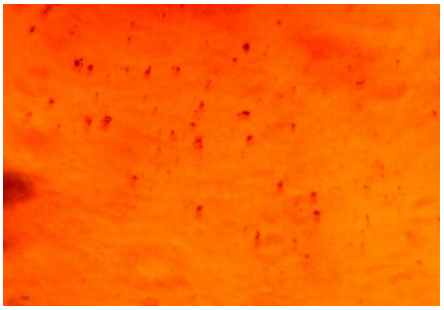


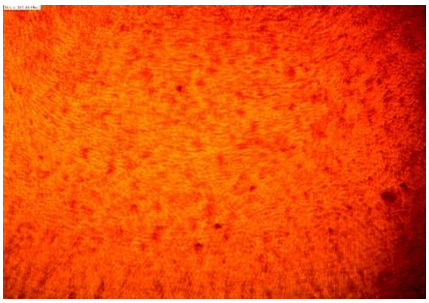
Fig 4: MTT Assay of PYR derivatives



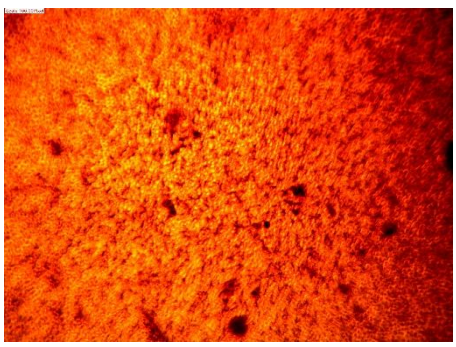
5-flurouracil (30 µg/ml)



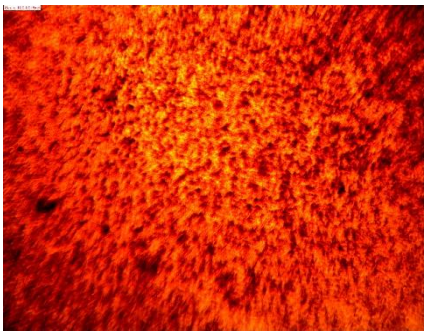
PYR-4 (30 µg/ml)



PYR-4 (20 µg/ml)



PYR-4 (20 µg/ml)



Control

^{1}H NMR Spectral analysis

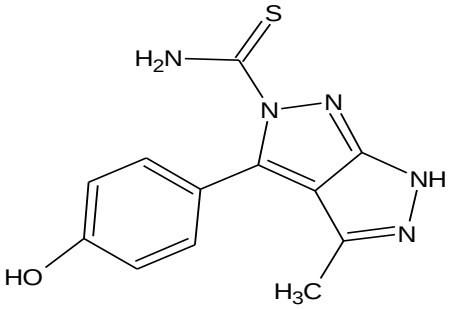


Table 7: ^{1}H NMR Spectral analysis of PYR 4

COMPOUND	^{1}H NMR
PYR 4	0.9055 (s,CH_3), 2.0919 (s, NH_2), 4.832 (s, OH), 7.0 (s, NH), 7.117-7.22 (m, aromatic proton)

Fig 5: ^{1}H NMR spectra of PYR 4

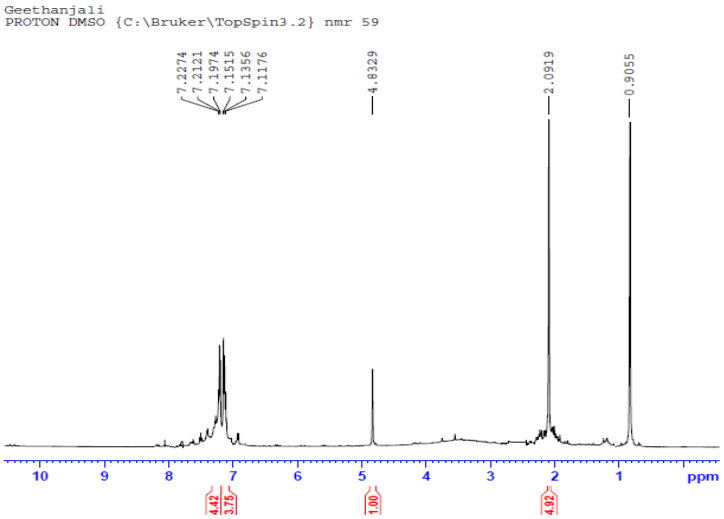


Table 8: Mass spectral analysis of PYR 4

COMPOUND CODE	MOLECULAR ION PEAK	PARENT PEAK
PYR 4	273.79	191.1

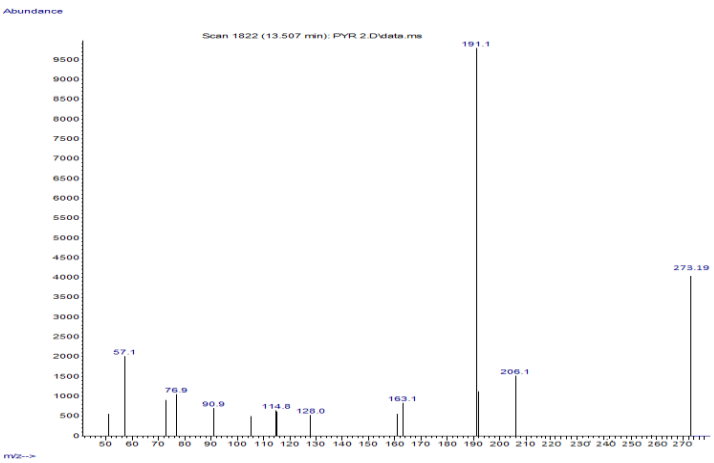


Fig 6: Mass spectra of PYR 4

CONCLUSION:

This research work was focused on the design, synthesis and pharmacological screening of new pyrazole derivatives. The work involved the preliminary screening of various pyrazole derivatives for their drug likeness, physicochemical properties, analysis of Lipinski rule of five, molecular docking studies and prediction of biological activities using various softwares. From the designed compounds, 10 compounds were selected for wet lab synthesis on the basis of dock score, PASS value and availability of chemicals. The synthesized compounds were subjected to in vitro screening for anticancer and anti-inflammatory activity. Among the 10 derivatives, PYR2, PYR 4, PYR 6 exhibited good activity.[27] So these analogues can be considered as future leads and can be subjected to pharmacological screening in detail for further evaluation.

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