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Synthesis, Spectral Analysis and Anticancer Evaluation of Novel Pyrazoline Derivatives

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ABSTRACT

The novel Pyrazoline derivatives were prepared by cyclisation of substituted chalcone derivatives in the presence of 2, 4 dinitro phenyl hydrazine hydrate. Structural elucidation of all synthesized derivatives were done by spectral analysis (IR , NMR and Mass spectroscopy). All synthesized derivatives screened for their anticancer activities by MTT assay and these prepared derivatives exhibits promising anticancer activities.

Keywords: Chalcones , 2,4 dinitro phenyl hydrazine hydrate , spectral analysis , anticancer activity , MTT assay.

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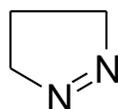
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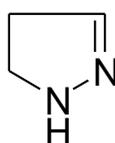
INTRODUCTION

Heterocyclic compounds have gained much importance in medicinal chemistry due to its presence in large number of pharmacologically active moieties. Among the five membered heterocyclic containing two hetero atoms in its ring structure , pyrazole is one of the most important one as large variety of biological activities have been reported for various pyrazole derivatives. Pyrazoline is dihydropyrazole, a five membered heterocyclic compound containing two nitrogen atoms in adjacent positions and possessing only one endocyclic double bond. Depending on the position of the double bond three forms of pyrazoline are possible. These are 1-pyrazoline, 2-pyrazoline and 3-pyrazoline. Among all the pyrazolines, 2-pyrazoline has gained attraction and is frequently studied one [1-42].

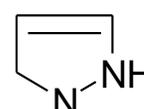
Increasing evidence suggests that pyrazoline derivatives posses a broad spectrum of biological activities such as tranquillizing, muscle relaxant, antidepressant, anticonvulsant, psychoanaleptic , antimycobacterial and antihypotensive activities[1-42].



1-Pyrazoline



1,2-pyrazoline

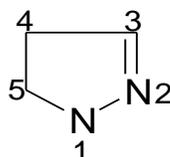


3-pyrazoline

The above three represents the tautomeric forms of pyrazoline structures.

Nomenclature:

The application of present heterocyclic nomenclature to pyrazolines requires that nitrogen atoms be numbered one and two in each structure. Substituted 1-pyrazolines are numbered to produce the lower of two possible numbers for substituent group locants, or in the case of complicated structures to produce the simplest name consistent with clarity of meaning. Numbering of the 2-pyrazolines begins with the amino nitrogen and 3-pyrazolines are numbered to obtain for the double bond the lower of the two possible numbers. Thus, here this structure will be referred as[1-42],



1,2-Pyrazoline

Chemistry:

The chalcones were synthesized from an aldol condensation between benzaldehyde and an acetophenone by Aldol-condensation reaction. An aldol condensation is an organic reaction in which an enol or an enolate ion reacts with a carbonyl compound to form a β -hydroxyaldehyde or β -hydroxyketone, followed by a dehydration to give a conjugated enone. The pyrazoline derivatives were synthesized from chalcones by refluxing with 2,4-dinitro phenyl hydrazine in ethanol.

MATERIALS AND METHOD

The chemicals used in the present work were AR grade and LR grade, purchased from Ranbaxy, Merck, S.D. Fine chemicals and Research Lab and used as received. The list of chemicals used were 4-hydroxyacetophenone, 4-methylacetophenone, 2-Acetyl furan, 4-methoxy benzaldehyde, 4-fluoro benzaldehyde, sodium hydroxide, 2,4-dinitro phenyl hydrazine, ethyl-acetate, HCl, ethanol, conc.HCl, light paraffin oil, pet ether, glacial acetic acid, chloroform. The water used was double distilled deionized water. All the compounds showed satisfactory elemental analysis for C, H & N.

Identification and characterization of synthesized products:

The synthesized compounds were scaled for yield and purified by recrystallization with suitable solvent system. The purified compounds were assigned for physical constant determination and further subjected for spectral analysis like thin layer chromatography, Infrared spectroscopy, Nuclear magnetic resonance spectroscopy.

1. Melting point determination:

The melting point of the synthesized compounds were determined using Veego VMP-I melting point apparatus and recorded in degree Celsius.

2. Thin layer chromatography:

Thin layer chromatography was performed on percolated silica gel plates with suitable solvent system. The R_F values were recorded accordingly.

3. Infrared spectroscopy:

The infrared spectra for the synthesized compounds were recorded using JASCO-FTIR 8400 spectrophotometer using potassium bromide pellet technique.

4. Nuclear magnetic resonance spectroscopy:

^1H NMR and ^{13}C NMR spectra of the synthesized compounds were taken using tetramethyl silane as an internal standard. ^1H NMR spectra were recorded with DMSO and CDCl_3 as a solvent and the chemical shift data were expressed as δ values relative to TMS. ^{13}C NMR spectra were recorded

with DMSO and CDCl_3 as a solvent and the chemical shift data were expressed as δ values relative to TMS. (Samples were sent to Chemistry department, Shivaji University, Kolhapur. For NMR analysis.)

Step-1. Synthesis of chalcones:-

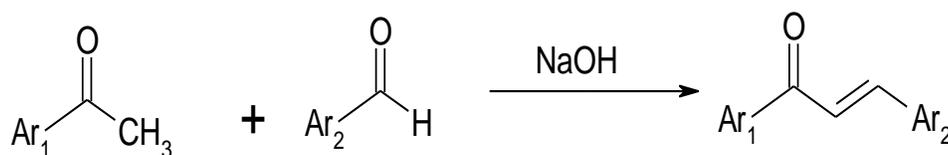
Equimolar quantity of substituted acetophenone (0.01 mol) and substituted aldehyde (0.01 mol) was dissolved in ethanol (10ml) under stirring & aq. NaOH (30 %) was added dropwise. The reaction mixture stirred at room temperature using magnetic stirrer & kept for few hour. The reaction mixture was diluted with water & acidified with HCl . The separated solid was filtered and recrystallised from ethanol.

Step-2. Synthesis of pyrazoline derivatives:-

A mixture of chalcones (0.01 mol) , 2,4-dinitrophenyl hydrazine reagent dissolved in ethanol. The resulting mixture was refluxed for 2 or 3 hours. The resulting mixture was poured into ice water. The separated solid were filtered and recrystallised from ethanol.

EXPERIMENTAL DESIGN :

Step 1



Step 2

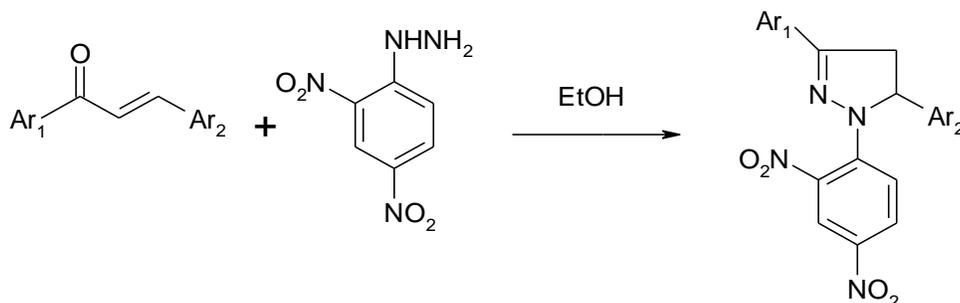


Table 1 : Newly Synthesized pyrazoline containing functional group

Compound	Ar ₁	Ar ₂
A		
B		

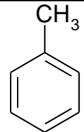
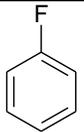
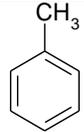
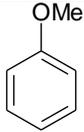
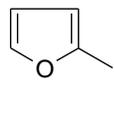
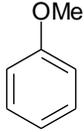
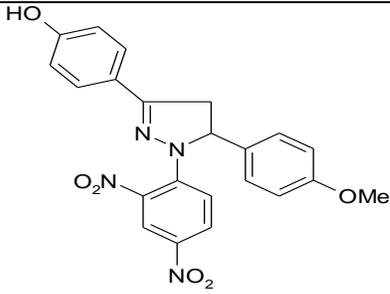
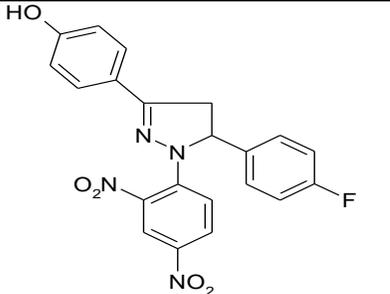
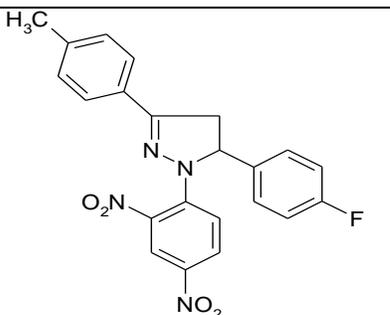
C		
D		
E		

Table 2: List of compounds synthesized

Compound	Chemical name	Structure
A	1(2,4-dinitrophenyl)3phenol-5(4-methoxyphenyl)pyrazoline	
B	1(2,4-dinitrophenyl)3phenol-5(4-fluorophenyl)pyrazoline	
C	1(2,4-dinitrophenyl)3(4-methylphenyl)-5(4-fluorophenyl)pyrazoline	

D	1(2,4-dinitrophenyl)3(4-methylphenyl)-5(4-methoxyphenyl)pyrazoline	
E	1(2,4-dinitrophenyl)3(2-furyl)-5(4-methoxyphenyl)pyrazoline	

Table 3: data for the Physicochemical compounds (1a-1e) (Chalcones)

CompNo.	Molecular Formula	Molecular Weight	M.P. range (°C)	Mobile Phase	*R _F value
1a	C ₁₆ H ₁₄ O ₃	254	120-122	Pet-ether : ethylacetate (5:5)	0.73
1b	C ₁₅ H ₁₁ O ₂ F	241.99	100-102	Pet –ether : ethylacetate (5:5)	0.84
1c	C ₁₆ H ₁₃ OF	239.99	90-92	Ethanol:ethylacetate:acetic-acid (5:4:1)	0.79
1d	C ₁₇ H ₁₆ O ₂	252	80-82	Ethanol:ethylacetate:acetic-acid (5:4:1)	0.65
1e	C ₁₄ H ₁₂ O ₃	228	85-87	Ethanol:ethylacetate:acetic-acid (5:4:1)	0.84

Table .3: Physicochemical data for the synthesized compounds (Pyrazolines)

Comp. No.	Molecular Formula	Mol. Weight	M.P. range (°C)	Mobile Phase	*R _F value	React ⁿ time, Hrs.
2a	C ₂₂ H ₁₆ N ₄ O ₆	432	158-160	Pet-ether : ethylacetate (5:5)	0.58	1.5
2b	C ₂₁ H ₁₃ N ₄ O ₅ F	419.99	140-142	Pet –ether : ethylacetate (5:5)	0.74	2
2c	C ₂₂ H ₁₅ N ₄ O ₄	417.99	130-132	Ethanol:ethylacetate:acetic-acid (5:4:1)	0.62	1.5
2d	C ₂₃ H ₁₈ N ₄ O ₅	448.99	110-112	Ethanol:ethylacetate:acetic-acid (5:4:1)	0.70	2
2e	C ₂₀ H ₁₄ N ₄ O ₆	424.99	100-102	Ethanol:ethylacetate:acetic-acid (5:4:1)	0.90	2

1(2,4-dinitrophenyl)3phenol-5(4-methoxyphenyl)pyrazoline (A)

IR (KBr,cm⁻¹): 3354 (OH str phenolic), 1601 (C-N), 1514, 1449,1338 (Aromatic C=C str), 1293, 1006 (C-O str),1514(N-O str).

¹H-NMR (δ ppm): 6.764-7.736 (, Ar-H), 3.294 (OCH₃), 2.410(OH)

¹³C-NMR (δ ppm): 128.35(C=N), 130.41(C in Aromatic ring), 29.71(C-N), 95.41(C-O), 55.34(C-N), 11.34(CH₃)

1(2,4-dinitrophenyl)3phenol-5(4-fluorophenyl)pyrazoline (B)

IR (KBr,cm⁻¹): 3348 (OH str phenolic), 2923 (-CH str), 1607, 1512, 1442 (Aromatic C=C str), 1344 (NO₂),1202(C-N),1344(C-F), 1282(C-O).

¹H-NMR (δ ppm): 7.281-7.741(Aromatic ring), 2.410(OH), 7.751(Ar-F), 3.882(Ar-OH)

¹³C-NMR (δ ppm): C=N(127.41), 128.83(C in Aromatic ring),29.71(C-N),21.46(C-C), 56.27(C-F),11.34(CH₃)

1(2,4-dinitrophenyl)3(4-methylphenyl)-5(4-fluorophenyl)pyrazoline (C)

IR (KBr,cm⁻¹): 1042(C-F),1285(C-N),1536(Niro gp),1650(C=C),2309(C-H).

¹H-NMR (δ ppm):7.910-8.225(Aromatic ring), 1.239(CH₃)

¹³C-NMR (δ ppm):): 127.32(C=N), 130.41(C in Aromatic ring),29.71(C-N),21.46(C-C), 56.37(C-F), 11.44(CH₃)

1(2,4-dinitrophenyl)3(4-methylphenyl)-5(4-methoxyphenyl)pyrazoline(D)

IR (KBr,cm⁻¹): 1035(C-C),1644(C=C),1358(C-N),989(C-O).

¹H-NMR (δ ppm): 6.764-7.751(Aromatic ring), 3.291(OCH₃), 1.279(CH₃),

¹³C-NMR (δ ppm):): 123.11(C=N), 128.33(C in Aromatic ring),30.11(C-N),21.44(C-C), 11.23(CH₃)

1(2,4-dinitrophenyl)3(2-furyl)-5(4-methoxyphenyl)pyrazoline(E)

IR (KBr,cm⁻¹):1464(Nitro gp),1271(C-O-C), 1332(C-N), 1593(C=C), 1367 (C-H), 992(C-H) .

¹H-NMR (δ ppm): 6.742-7.742(Aromatic ring), 2.440(OCH₃)

¹³C-NMR (δ ppm): 123.11(C=N), 128.33(C in Aromatic ring),29.11(C-N),11.82(C-C), 22.16(CH₃), C-O(97.31)123.31(C=C)

BIOLOGICAL ACTIVITY:

Anticancer activity:

Materials and methods Chemicals and reagents :

All chemicals and solvents were obtained from commercial sources, purified and sterilized using standard procedures wherever required. The chemicals and reagents used were AR grade and LR grade, purchased from Ranbaxy, Merck, S.D. Fine Chemicals and Research Lab and used as received. Trypsin, Trypan blue, DPBS (Dulbecoo's phosphate buffer saline), EDTA, 3-(4,5-

dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT Salt), dimethylsulfoxide (DMSO), methanol, ethanol.

Equipment and instruments: Hemocytometer, cover slip, vials, Micropipettes, pipette tips, microscope, Tissue culture flasks, Flat bottomed 96-well micro titer plate (Laxbro manufacturing company, Pune), Deep freezer, Laminar flow clean air work station (Klenzaid's biochem devices ltd), Automatic CO₂ Incubator (NUAIRE-5100E), ELISA plate reader(EL X 800, Biotek).

Cell line used: Hep G2 (Human hepatoma cell line)

Culture Medium:

Hep G2 cell line was cultured in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L Na bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM Na pyruvate 90%, fetal calf serum 10%. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and was subcultured twice a week.

Preparation of test material: Stock solution:

All the synthesized compounds that are pyrazoline derivatives were dissolved in PBS so as to get the concentrations of 10 µM, 20 µM and 50 µM. In the preparation of stock solution 1% DMSO and was used in small volume in order to achieve complete solubilization of synthesized compounds and drugs.

Positive controls

5-Flurouracil for Hep G-2 cell line

Method utilized – MTT assay In-vitro cytotoxicity assay Preparation of Cell suspension for assay: The desired human cancer cell line were grown in multiple TCFs at 37°C in an atmosphere of 5% in CO₂ and 90% relative humidity in complete growth medium to obtain enough number of cells as per requirement depending upon number of test samples.

DAY ONE

Cells were harvested by treatment with Trypsin- EDTA and added to complete growth medium to stop the action of trypsin. Cells were separated to single cell suspension by gentle pipetting action.

90µl of cell suspension was stained with 10µl of Trypan blue.

The viable cells were counted in a hemocytometer.

Viable cell density was adjusted depending upon the cell line using the medium containing 10% fetal bovine serum.

100µl of cell suspension was added into each well.

C for 24 hours in an atmosphere of 5% CO₂ and 90% relative humidity in a CO₂ incubator.

After 24 hours, the test material and positive controls were added.

DAY TWO - Addition of test materials

Added 100µl of stock solutions of the test materials along with positives controls & negative controls into these wells in the tissue culture plate.

The plates were incubated at 37°C for 48 hours (Hep G2) in an atmosphere of 5% CO₂ and 90% relative humidity. The cell growth was determined after 24 hours by MTT assay.

DAY THREE - MTT assay

20 µl of 5 mg/ml MTT was added to each well aseptically.

The plates were incubated for 4 hours in an atmosphere of 5% CO₂ and 90% relative humidity in a CO₂ incubator.

Media was removed CAREFULLY.

200 µl of DMSO was added and mixed well.

Optical density was recorded on ELISA reader at 570nm (background wavelength is 630nm) and then the data was recorded.

RESULTS AND DISCUSSION

The main objective of the study was to synthesize pyrazoline from aldehyde and ketone using as a starting material. Total five derivatives were synthesized in fairly good yield and yield of all derivatives were lies in the ranging from 68% to 82 %.

Thin layer chromatography was used to checking the completion of reaction as well as purity of all final products.

The structural characterization of synthesized compounds were done by interpretation of IR, ¹HNMR, ¹³CNMR & GCMS All the compound exhibited satisfactory IR, NMR data.

The test compounds were screened for anticancer activity by MTT assay.

ANTICANCER ACTIVITY

Compound was screened for anticancer activity against Hep G2 (Human hepatoma cell line).

Percent Cytotoxicity = Reading of control - Reading of treated cells / Reading of control X 100

Table 3 : anticancer activity of synthesized compound against Hep G2 (Human hepatoma cell line)

Compound	% inhibitory (µM)		
	10	20	50
A	15.55	19.25	21.55
B	6.55	7.25	10.12
C	16.28	23.12	36.55
D	22.4	29.25	36.25
E	23.51	28.78	34.25
5 FU	17.27	29.11	40.12

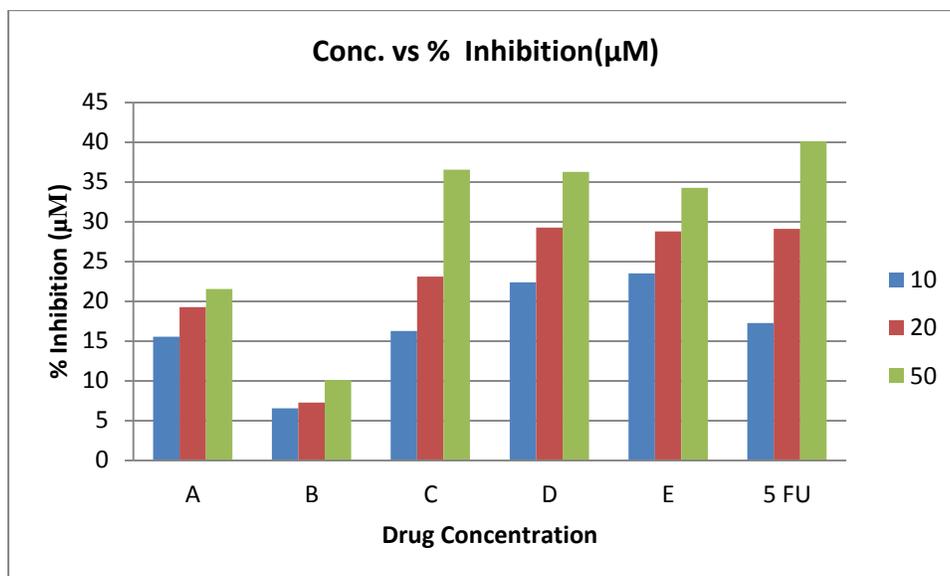
5FU : 5 Fluorouracil

Figure 1: Drug concentration vs percent inhibitory

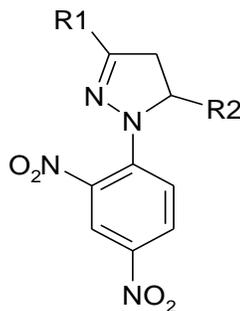
Generally, as shown in table , the prepared synthetic compound (A-E) displayed moderate to good inhibition activities against Hep G-2 cell line. Notably, the compounds C,D,E exhibited significant inhibitory activities against Hep G-2 cell line with 16.28 , 22.4 , 23.51% growth inhibition at 10µM /mL concentration compared to the positive control 5-FU (17.27%). The activity is due to the substituted nitro group on C -2 & C-4 position on aromatic group.

CONCLUSION

The synthetic scheme reported in this study design is novel example in heterocyclic synthesis. The synthesis of pyrazoline derivatives was carried out in two steps. These are as

1. Formation of Chalcone
2. Cyclisation to form pyrazolines

In firstly , when substituted aldehyde treated with substituted ketone to form chalcone. Further treated with suitable 2,4- dinitro phenyl hydrazine hydrate to form corresponding pyrazolines. The yield of all derivatives were lies in the ranging from 68% to 82 % . All synthesized compounds were meeting the expected spectral data. General structure confirmed from the collected spectral data is as follows,



All synthesized compounds characterized by spectral analysis.

All synthesized compounds are screened for anticancer activity and compared with standard drug. From the results it can be concluded that the modified pyrazoline shows remarkable anticancer activity.

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