Synthesis, Characterization and Biological Evaluation of 5-(2-Nitrophenyl)-1*H*-Pyrazole Derivatives as Putative Antiproliferative Agents

A Project Report submitted to the Central University of Punjab

For the award of

Master of Science (Chemical Science)

In

Medicinal Chemistry

By
Geetika Saini
(16mscchs05)

Supervisor

Dr. Raj Kumar

Department of Pharmaceutical Sciences and Natural Products
School of Basic and Applied Sciences
Central University of Punjab, Bathinda
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CERTIFICATE

I declare that the dissertation/thesis entitled "SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF 5-(2-NITROPHENYL)-1H-PYRAZOLE DERIVATIVES AS PUTATIVE ANTIPROLIFERATIVE AGENTS" has been prepared by me under the guidance of Dr. Raj Kumar, Associate Professor, Head of Department, Department of Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab. No part of this project report has formed the basis for the award of any degree or fellowship previously.

Geetika Saini
(16mscchs05)

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School of Basic and Applied Sciences

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Date:

CERTIFICATE

I certify that GEETIKA SAINI has prepared her project report entitled "SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF 5-(2-NITROPHENYL)-1H-PYRAZOLE DERIVATIVES AS PUTATIVE ANTIPROLIFERATIVE AGENTS", for the award of M.Sc. degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department of Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab.

Dr. Raj Kumar

Associate Professor, Head of Department

Department of Pharmaceutical Sciences and Natural Products

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Central University of Punjab

Bathinda-151001

Date:

ABSTRACT

Synthesis, Characterization and Biological Evaluation of 5-(2-Nitrophenyl)-*1H*-Pyrazole Derivatives as putative Antiproliferative Agents

Name of the student : Geetika Saini
Registration Number : 16mscchs05
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Name of Supervisor : Dr. Raj Kumar
Name of Department : Pharmaceutical Sciences and Natural Products
Name of School : School for Basic and Applied Sciences

Key Words : cancer, topoisomerase, pyrazole, antiproliferative, synthesis

ABSTRACT

Pyrazoles are known to exhibit various biological activities like antibacterial, antiprotozoal, anticonvulsant, analgesic, anti-inflammatory, antiviral and antiproliferative. An attempt has been made to synthesize substituted pyrazoles. Their antiproliferative activity was determined by performing MTT assay on MDA-MB 231 cell line (breast cancer). The compounds were further docked into topoisomerase 1 and 2.

Geetika Saini
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Dr. Raj Kumar

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Geetika Saini

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LIST OF ABBREVATION

S. No.	Full Form	Abbreviation
1.	Human topoisomerase	htopo
2.	Nuclear magnetic resonance	NMR
3.	Cyclooxygenase	COX
4.	World Health Organization	WHO
5.	Multi-drug resistance	MDR
6.	Epidermal growth factor receptor	EGFR
7.	Thin layer chromatography	TLC
8.	Fourier Transform Infrared	FT-IR
9.	Protein data base	PDB
10.	Doublet	d
11.	Singlet	s
12.	Multiplet	m
13.	Coupling constant	J
14.	Parts per million	ppm
15.	Hertz	Hz
16.	Milliliter	mL
17.	Milligram	Mg
18.	Millimole	mM
19.	Micromole	µM
20.	Melting point	mp

21.	Concentration	Conc.
22.	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide	MTT
23.	Standard deviation	SD
24.	Carbonic Anhydrase	CA
25.	Fibroblast growth factor	FGF
26.	4',6-diamidino-2-phenylindole	DAPI
27.	Deoxyribonucleic acid	DNA
28.	Microtubulin	MT
29.	Dimethyl sulfoxide	DMSO

Chapter-1

Introduction

Introduction

Cancer has become as prevalent threat to the human existence as are the environmental and energy crisis. With over 100 types of cancers, no part of the human body is immune to cancer. We can define Cancer as an uncontrolled growth of abnormal cells called the tumor cells or the malignant cells, anywhere in the body which infiltrate normal tissues. The National institute of Cancer Prevention and Research reports that approximately 2.5 million people in India suffer with cancer and every year around 7 lakh new cases are being reported (Nandakumar, 2009). The most common types of cancer diagnosed among women include breast, ovary, lip and oral cavity, lung and cervix. Lung, colorectal, pharynx, stomach, head and neck, and liver cancer are on the top of the list among men. Oral cavity and lung cancer in males and cervical and breast cancer cause over 50% of cancer related deaths in India. Cervical cancer leads to death of a women every 8 minutes in India(Reddy andGupta, 2004). Tobacco is the root cause of around 20 per cent of global cancer deaths and nearly 70 per cent of global lung cancer. With 13 per cent of all new cancer cases reported and cause of 19 per cent of cancer related deaths worldwide lung cancer is among the most widely spread cancers.

Cancer development can be attributed to various factors including environmental, life style and genetic or their combination. Exposure to toxic chemicals, ionizing radiations, pathogens are important triggers for cancer (Stewart andWild, 2017). Treatment for cancer is determined by the type and stage of cancer, which is generally a combination of surgery, chemotherapy or radiation therapy. Cancer can be treated by targeting various kinases (Aurora, Chk), enzymes (COX, CA), receptors (EGFR), pathways (Ras-Net) or genes which are instrumental in cancer development or existence of tumor cells. Since the numbers of targets for cancer treatment are as vivid as types of cancer it is important to develop targeted chemotherapy which makes it specific, less toxic and has lesser instance of resistance development.

Chapter-2

Review of Literature

2. Review of Literature

2.1 Pyrazole as a pharmacologically significant scaffold

Pyrazoles constitute an important class of heteroaromatic ring systems that exhibit a variety of pharmacologically important properties (Anuta, Nitulescu *et al.*, 2014) such as antibacterial (Tanitame, Oyamada *et al.*, 2004), antifungal (Sun andZhou, 2015), analgesic (Gokulan, Jayakar *et al.*, 2012), anti-diabetic (Faidallah, Khan *et al.*, 2011), anticonvulsant (Abdel-Aziz, Abuo-Rahma *et al.*, 2009), anti-inflammatory (Alam, Marella *et al.*, 2013), antiviral (El-Sabbagh, Baraka *et al.*, 2009), anti-tuberculin (Castagnolo, De Logu *et al.*, 2008), anti-malarial (Kumar, Kumar *et al.*, 2006), anti-proliferative and cytotoxic effects (El-Gaby, Ghorab *et al.*, 2018). Celecoxib, sulfaphenazole,CDPPB, linazolac, mepiprazole, and rimonabant shown in figure 2.1 are some of the pyrazole-based drugs which are in use and available in the market (Kamel, 2015).

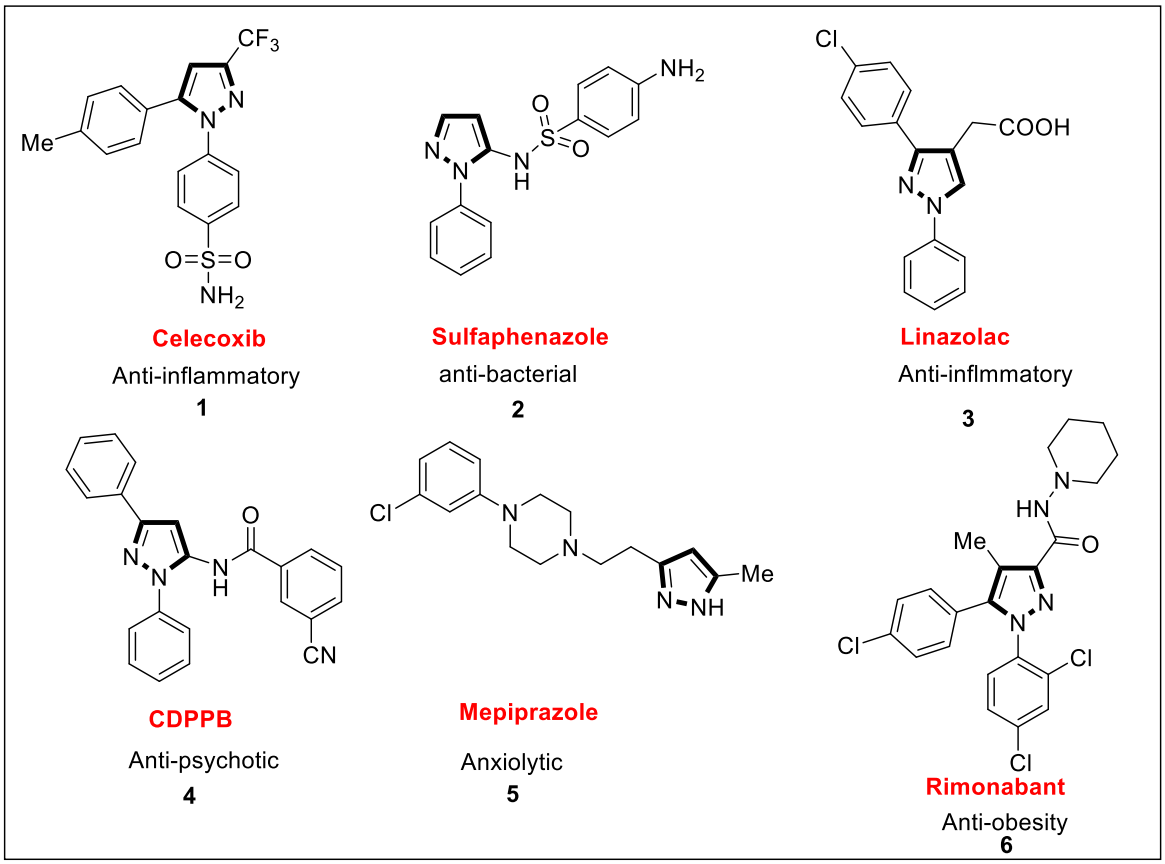


Figure 2.1 Pyrazole based Drugs

2.2 Pyrazole as an anti-cancer moiety

The anticancer activity of pyrazoles and their derivatives is due to their ability to inhibit topoisomerase I/II (TOPO I and II), epidermal growth factor receptor (EGFR), telomerase, vascular endothelial growth factor, aurora-A kinase, Janus kinase 2 (JAK2), c-Met, anaplastic lymphoma kinase (ALK), mTOR, cyclin-dependent kinases (CDK), B-Raf, p38 MAPK, glycogen synthase kinase-3, PI3K, Src family kinase, etc.

Crizotinib is an anti-cancer drug which acts as an ALK and ROS1 inhibitor and is used for treatment of some non-small cell lung carcinoma (Williams, Watson *et al.*, 2000). Ruxolitinib is a JAK2 inhibitory drug used for the treatment of intermediate or high-risk myelofibrosis, which is a type of myeloproliferative disorder that affects the bone marrow, shown in figure 2.2 (Koca, Özgür *et al.*, 2013).

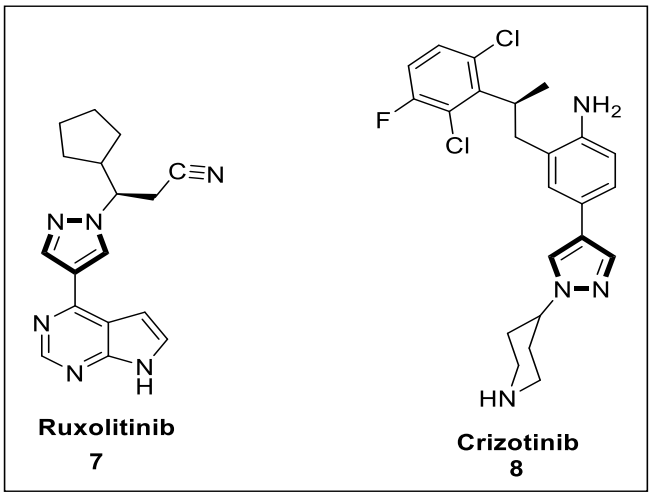


Figure 2.2 Pyrazole based Anticancer Drugs

Pyridine derivatives of pyrazole like **9** and **10** shown in figure 2.3 exhibit topoisomerase I and II inhibition and have cytotoxic effect on human cancer cell lines (Abdallah, Gomha *et al.*, 2017).

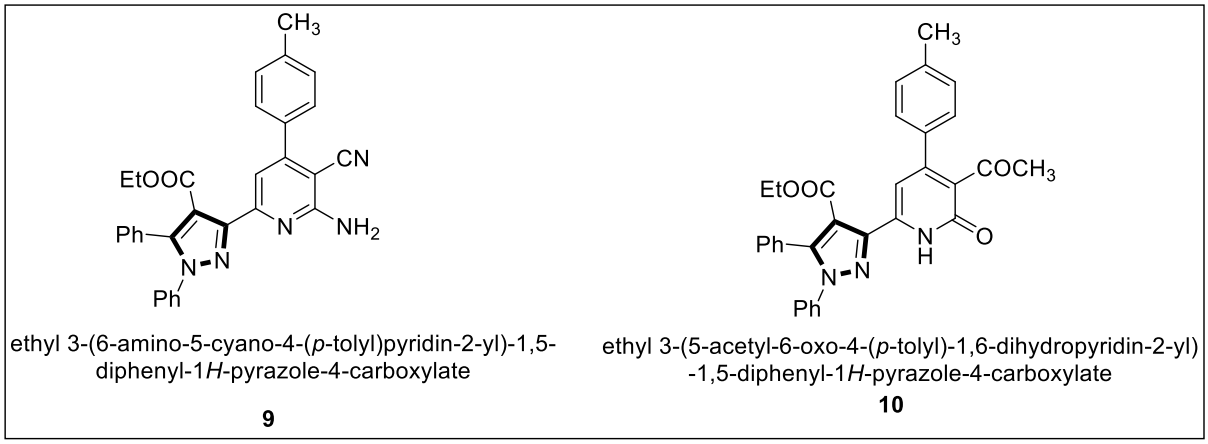


Figure 2.3 Pyridine derivatives of pyrazole

Pyrazole derivatives of thiosemicarbazide **11**, thiourea **12**, thiadoazole **13**, carbothioamide **14** shown in figure 2.4 are potent anticancer agents(El-Gaby, Ghorab *et al.*, 2018) .

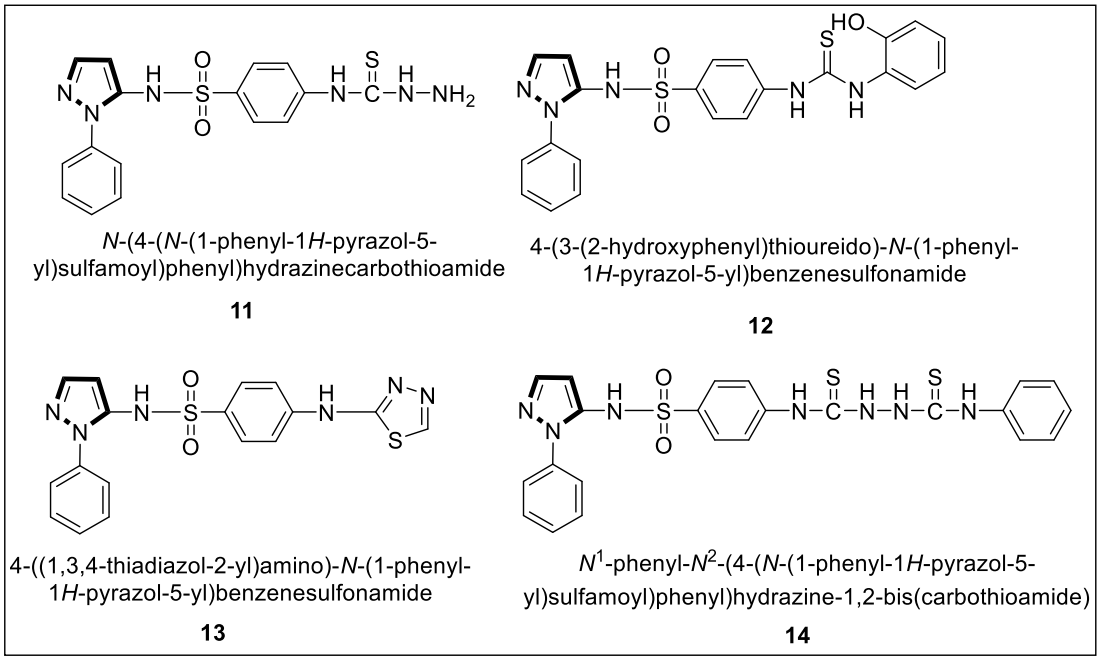


Figure 2.4 Potent anticancer agents

Quinoline based chalcones **15** and dihydro pyrazole **16** shown in figure 2.5 exhibit promising anticancer activity along with antifungal, antibacterial and antiprotozoal activity. They can serve as scaffolds for further development of more potent drugs (RamírezePrada, Robledo *et al.*, 2017).

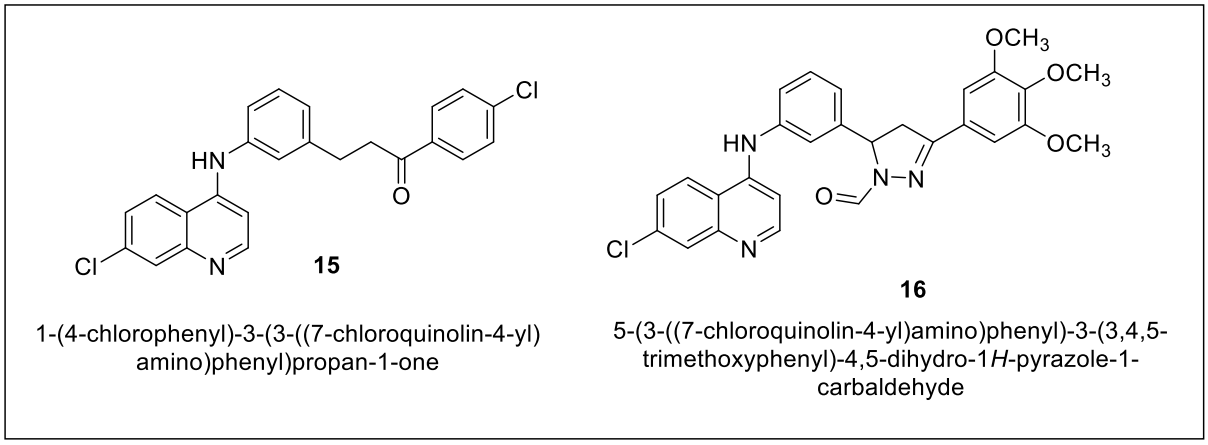


Figure 2.5 Quinoline based chalcones and dihydro pyrazole

Pyrazolo-quinoline derivatives like **17, 18, 19** and **20** shown in figure 2.6 have been reported to exhibit cytotoxicity against cancer cells derived from human liver, alveolar adenocarcinoma epithelial, colon and cervix (Dev, Poornachandra *et al.*, 2017).

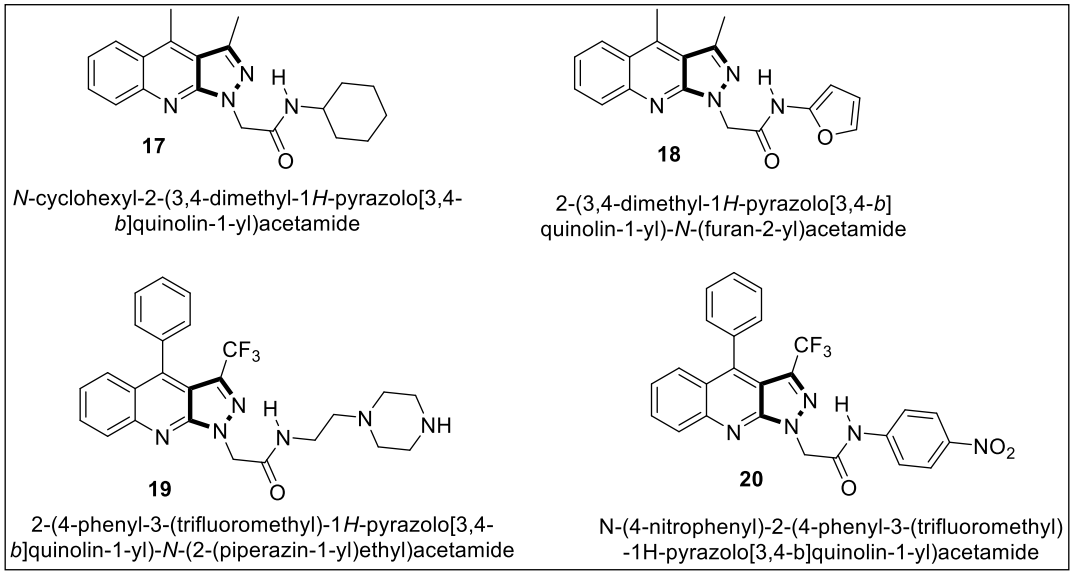


Figure 2.6 Pyrazolo-quinoline derivatives

2.2.1 Pyrazole as COX Inhibitors

Cyclooxygenase COX is an important enzyme in inflammation and is reported in solid malignancies. COX-2 inhibition is an approach in cancer treatment as inflammation has been reported to facilitate release of growth factors like epidermal growth factor (EGF) and fibroblast growth factor (FGF). Pyrazolo pyrimidine derivatives **21, 22** and **23** shown in figure 2.7 are reported to exhibit cytotoxicity equivalent to that of cisplatin and suppressed COX-2 protein expression(Abd El Razik, Mroueh *et al.*, 2017) .

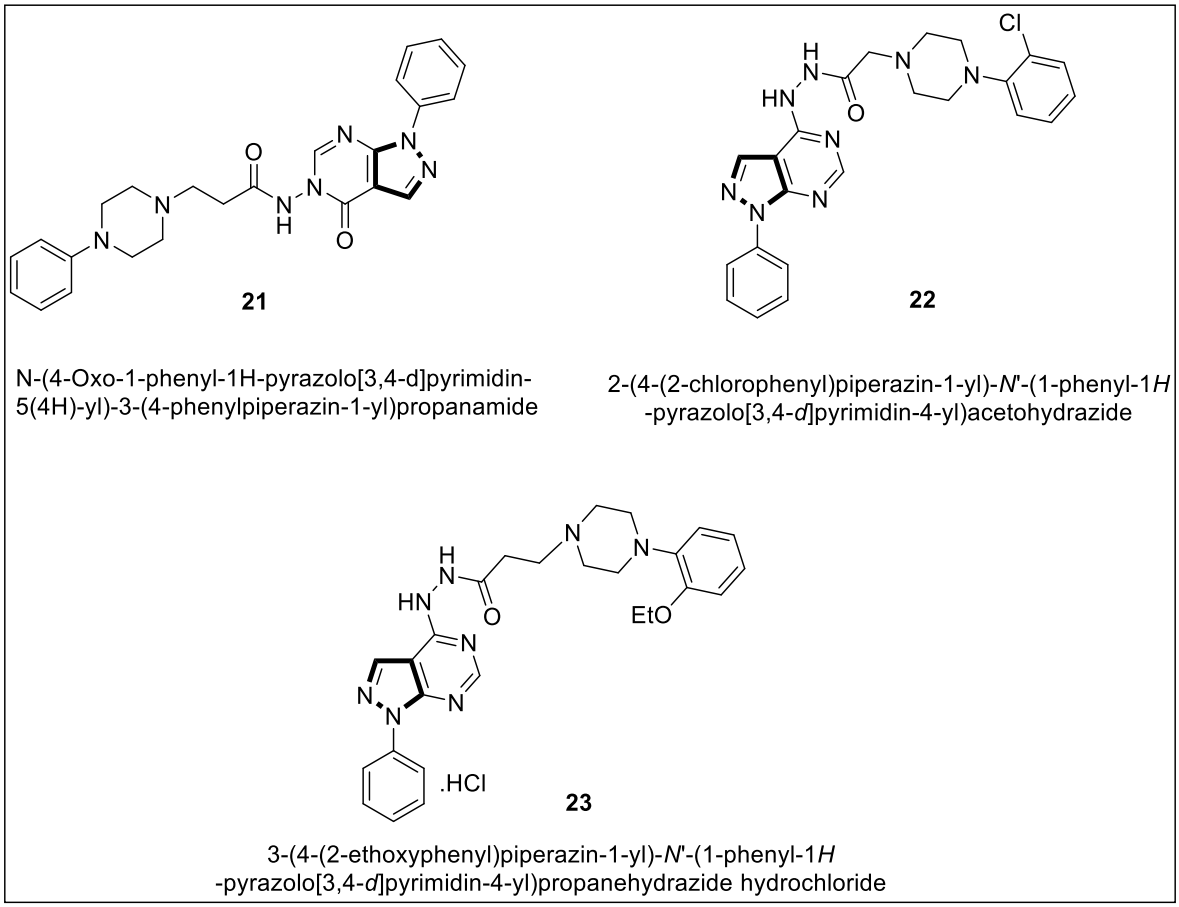


Figure 2.7 Pyrazolo pyrimidine derivatives

2.2.2 Pyrazole as Carbonic Anhydrase Inhibitors

Carbonic anhydrases are zinc metalloenzymes which catalyze reversible hydration of carbon dioxide. Fifteen different isoforms are found in humans, which belong to α-CA class and are involved in processes like pH homeostasis, ion transport, respiration, gluconeogenesis, bone resorption, renal acidification, formation of cerebrospinal fluid and gastric acid. Inhibition of these proteins is hence used as a therapeutic pathway for prevention of diseases like glaucoma, neurological disorders including epilepsy and altitude disease, obesity etc. Two membrane associated CAs, (CA IX and CA XII) have been identified, cloned and sequenced for their role in hypoxic tumors. CA IX controls cell proliferation, differentiation, and protects the integrity of stomach mucosa. Under hypoxic condition, CA IX is expressed drastically and has been found in carcinoma cells derived from organs like esophagus, lung, kidney, colon, rectum, breast, cervix, head and neck, and bladder (Winum, Rami *et al.*, 2008).

Taking COX-2 selective inhibitor celecoxib and valdecoxib as lead compounds shown in figure 2.8, a novel series of sulfonamide bioisoster has been developed. Substitution of pyrazole or isoxazole rings with Pyrazoline was attempted due to various biological activities reported for the same. The reported sulfamates exhibited potency equivalent to celecoxib against hCA IX while potency against hCA I, II, IV involved in glaucoma was higher (Nocentini, Moi *et al.*, 2018).

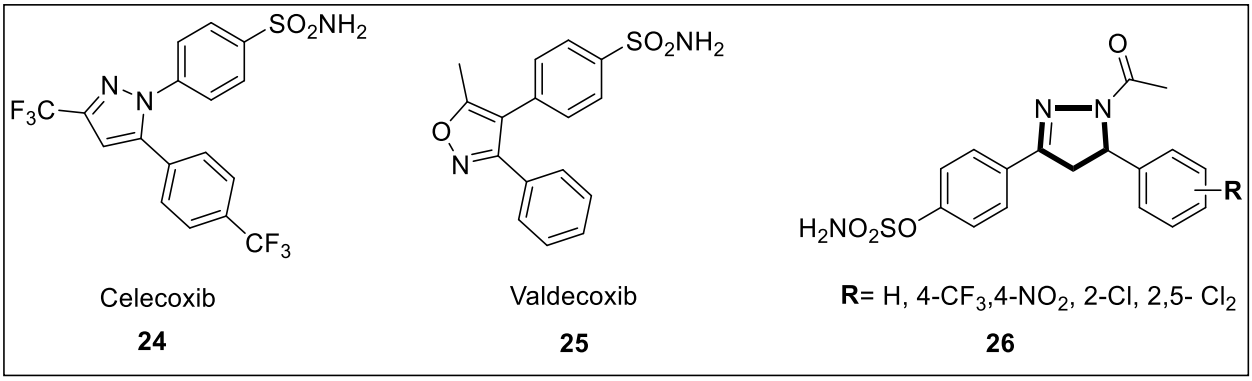


Figure 2.8 Pyrazole containing Carbonic Anhydrase Inhibitors

2.2.3 Pyrazole as Aurora Kinase Inhibitors

Three Aurora kinases A, B and C are found in mammals which have important roles in mitosis and completion of cell division. Aurora A plays role in centrosome maturation and separation, bipolar spindle assembly and mitotic entry and aurora B is required for accurate chromosome segregation and cytokinesis, while the role of aurora C is unclear (Vader andLens, 2008). Tumorigenesis and genetic instability is induced by over expression of aurora A, as it disrupts proper assembly of the mitotic checkpoint complex. It has been found in tumors such as breast, colorectal, ovarian as well as glaicoma and hence an attractive strategy for cancer therapy involves inhibition of aurora A. Four different scaffolds have been identified to exhibit aurora A kinase inhibition, having following cores, (**27**) 1,4,5,6-tetrahydropyrrolo[3,4-c] pyrazole; (**28**) pyrrolo[2,3-b] pyrimidine; (**29**) Quinoline; (**30**) 2-anilino-diamino-pyrimidine shown in figure 2.9. Further owing to the various biological activities of benzamide which include anticancer, anti-inflammatory and antifungal activities N-phenyl-1H-pyrazole-4-carboxamide and N, 1, 3-triphenyl-1H-pyrazole-4-carboxamide derivatives were synthesized shown in figure 2.10. The triphenyl derivatives exhibited potent activities against HTC116, MCF-7 cells and Aurora A kinase inhibitory activity. Molecular docking revealed potential binding between Aurora A kinase and compound **32** via two hydrogen bond and a π- cation interaction (Li, Lu *et al.*, 2012).

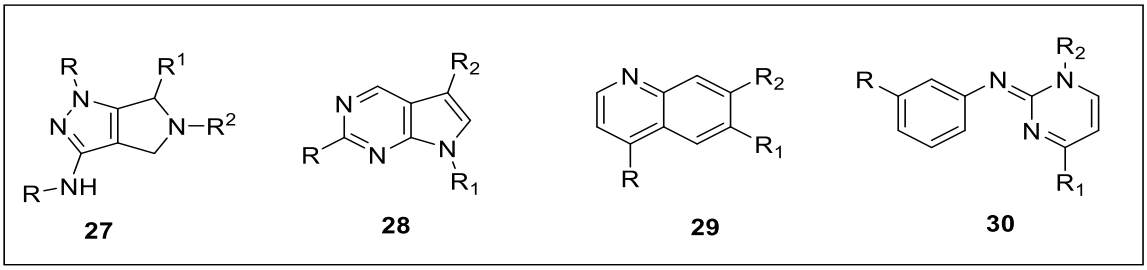


Figure 2.9 Scaffolds exhibiting aurora A kinase inhibition

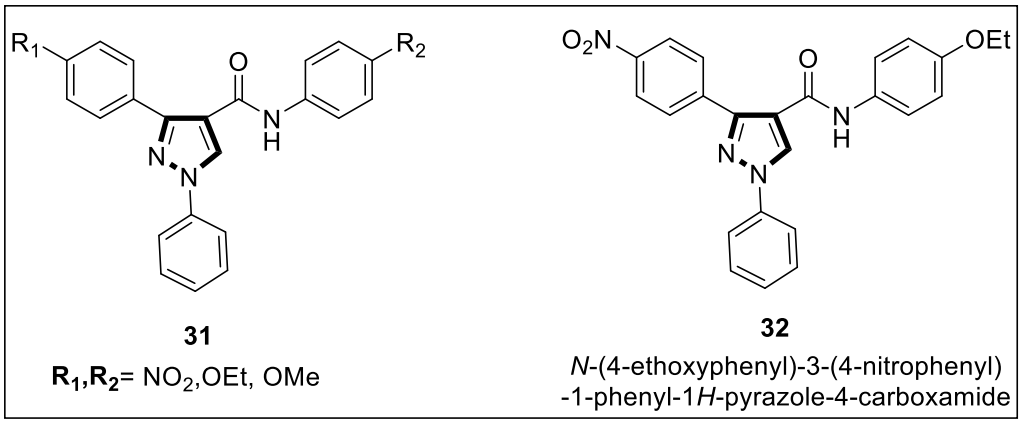


Figure 2.10 Triphenyl pyrazole carboxamide derivatives

2.2.4 Pyrazole as Apoptosis Inducers

Apoptosis or programmed cell death (PCD) or type 1 cell death is a terminal pathway in multicellular organisms and is responsible for a number of events like proliferation, homeostasis, differentiation, development and elimination of harmful cells as strategy to remove infected cells. Apoptotic signaling is important for preserving the balance between cell death and cell survival and for the maintenance of genome integrity. Various pro and anti-apoptotic activity inducing proteins have been described and their ratio is significant for regulation of cell death. Any dis-balance in the same leads to carcinogenesis due to reduction of apoptosis in malignant cells. Sensitivity of cancer cells to undergo apoptosis i.e. their apoptotic threshold can be increased by identifying mechanisms of apoptosis, its effecter proteins as well as the genes responsible for the same (Goldar, Khaniani *et al.*, 2015).

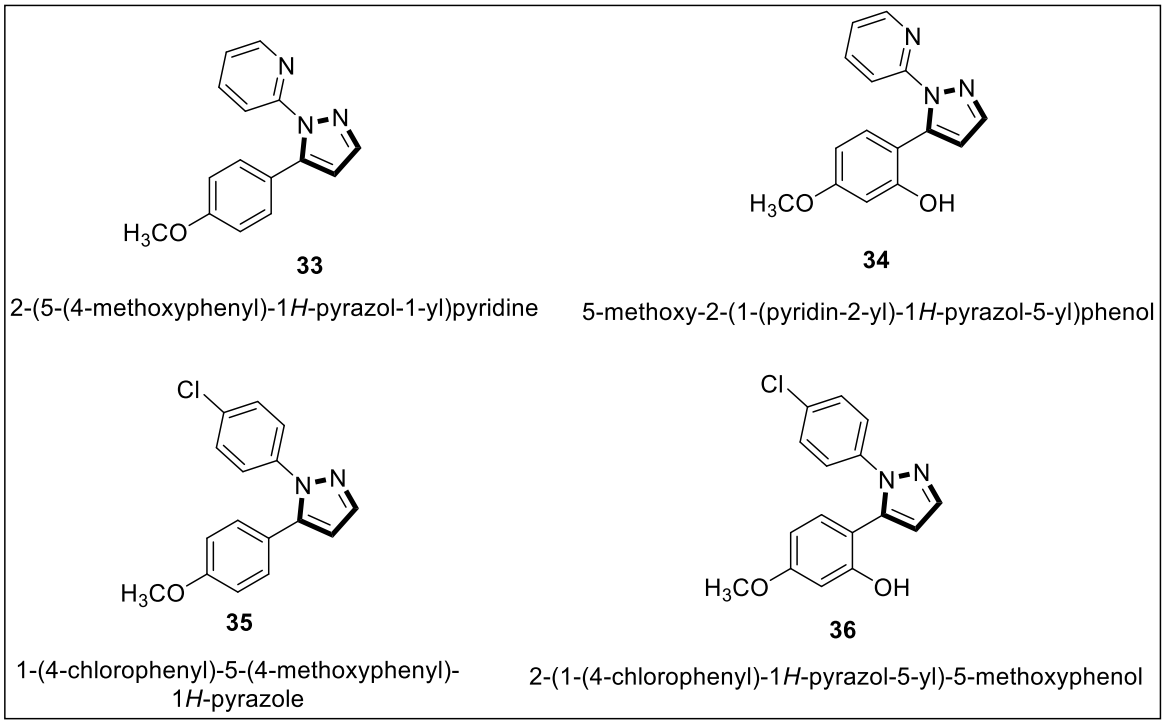


Figure 2.11 Pyrazole derivatives having apoptotic activity

Pyrazole derivatives **33, 34, 35** and **36** shown in figure 2.11 are active on human ovarian adenocarcinoma A2780 and murine leukemia P388 cells, but have no significant activity against human lung carcinoma. On testing apoptosis induction using DAPI staining and morphological analysis of nuclei on exposing equitoxic concentrations, all molecules showed a good concentration-response graph with maximum activity at 48h. The apoptotic activity shown was equal to or better than that of taxol and vincristine (Balbi, Anzaldi *et al.*, 2011).

Pyrazoline derivatives **37** and **38** shown in figure 2.12 decreases cell viability and inhibit colony formation in human bladder cancer cells and hence are promising candidates for development of anticancer drugs that can be used in long term treatment. Compound **38** has been reported to induce apoptosis (Tessmann, Buss *et al.*, 2017).

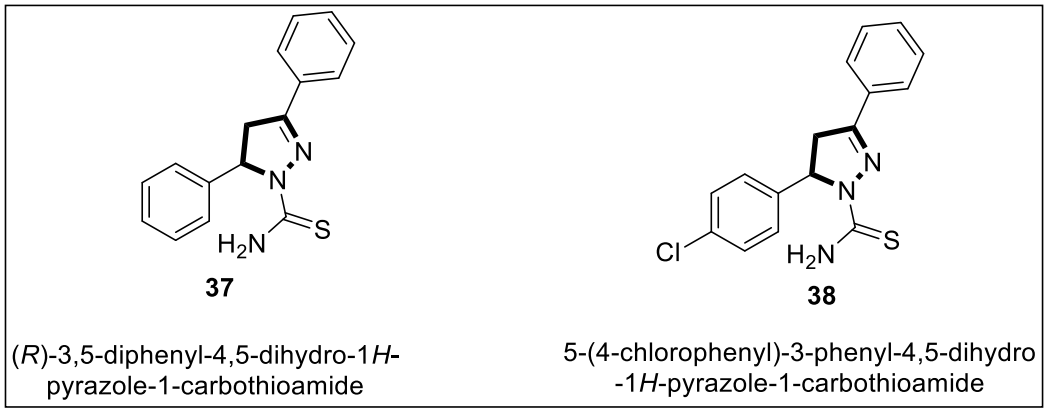


Figure 2.12 Pyrazoline derivatives having apoptosis inducing ability

2.2.5 Pyrazole as Checkpoint Kinase 2 Inhibitors

Checkpoint kinase 2 (Chk2) promotes cellular responses such as cell cycle regulation, DNA repair or apoptosis making it an important enzyme in the DNA damage-response pathway. It is activated by phosphorylation and two different arguments have been put forth for its role in current cancer therapies. It has been reported that certain cancerous cells due to over expression of Chk2 require high Chk2 for their survival and its inhibition is thus a way out leading to a apoptotic response. Further in normal cells, inhibition of Chk2 has led to radio protective effects prompting DNA repair by inhibiting IR induced p53 facilitated apoptosis.

Pyrazole-Benzimidazole conjugates have been reported as potent Chk2 inhibitors via structure based docking as analogues of 2-biarylbenzimidazoles. Compounds **39, 40, 41** and **42** shown in figure 2.13 have Chk2 inhibitory effects; additionally they potentiate the cytotoxicity of cisplatin and doxorubicin. **40** has antitumor effect against breast cancer bearing animals(Galal, Abdelsamie *et al.*, 2017).

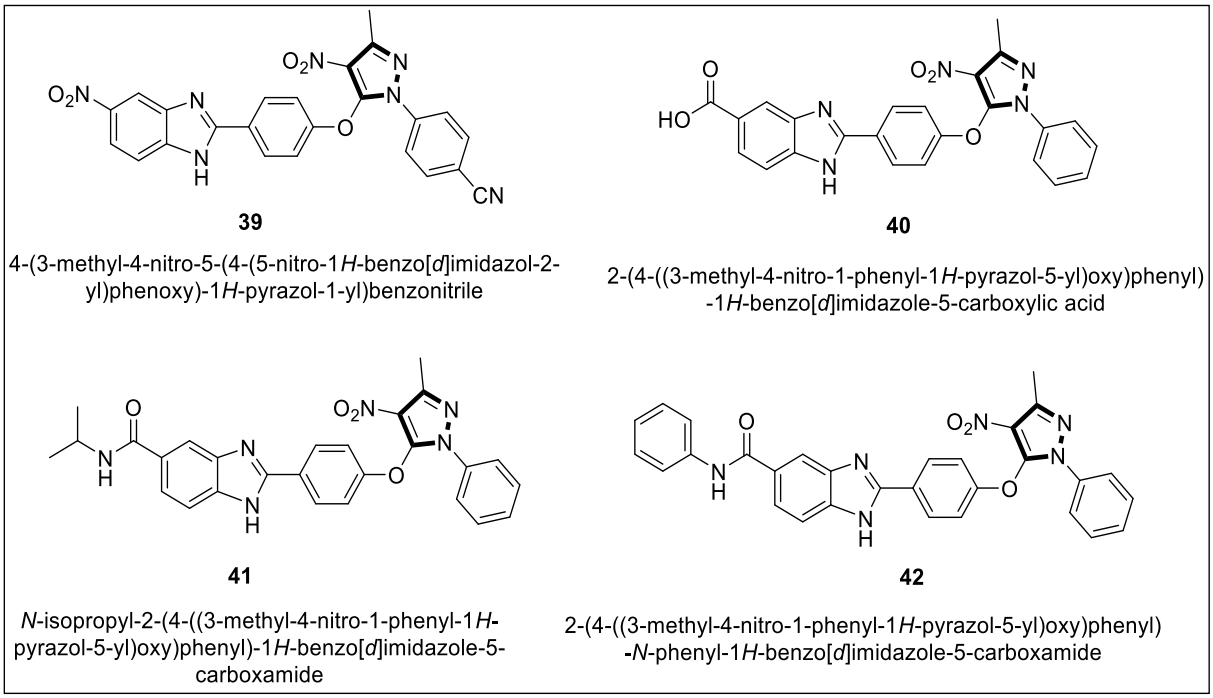
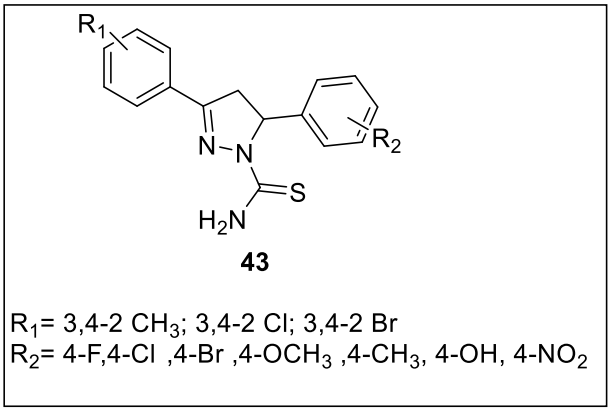


Figure 2.13 Pyrazole containing biarylbenzimidazole derivatives

2.2.6 Pyrazole as EGFR Inhibitors

Receptor protein tyrosine kinases and epidermal growth factor receptors have significant role in regulating tumor cell proliferation, differentiation, survival and apoptosis. EGFR is activated due to over expression, mutations or autocrine expression of ligand. EGFR are found to be over expressed in breast, ovarian, lung cancer and in hormone-refectory prostate cancer. And hence compounds which inhibit EGFR kinase on binding to its cognate ligand are of great interest as antitumor agents.

Owing to their inhibition of receptor tyrosine kinase (RTKs), protein tyrosine kinases (PTKs) and NADH oxidase which have important role in Tumorigenesis, thiourea and urea derivatives are of interest as anticancer agents. Their pyrazole derivatives shown in figure 2.14 are found to inhibit auto phosphorylation of EGFR kinases. These compounds exhibit high antiproliferative activity against MCF-7 (Lv, Li *et al.*, 2010).

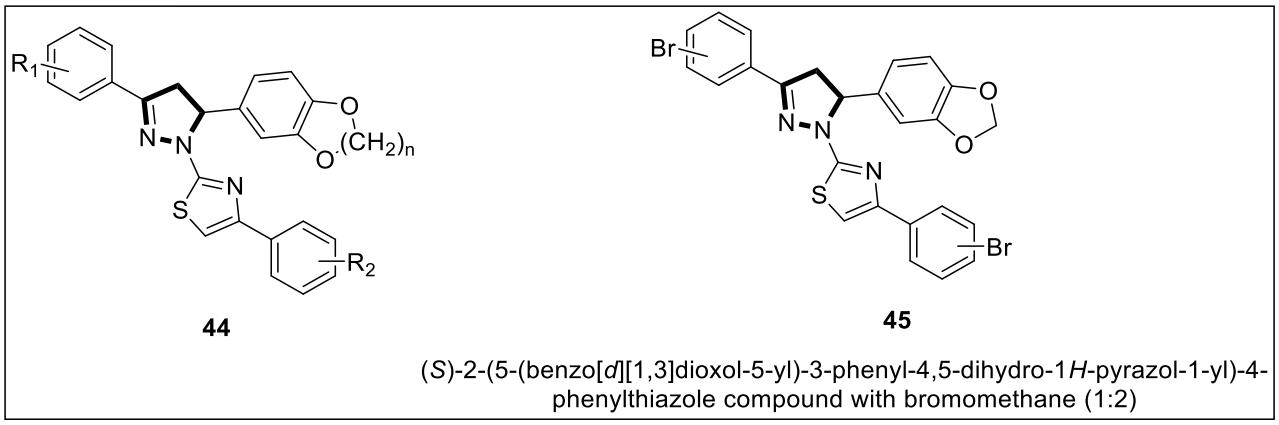


43

R_1= 3,4-2 CH_3; 3,4-2 Cl; 3,4-2 Br
R_2= 4-F,4-Cl ,4-Br ,4-OCH_3 ,4-CH_3, 4-OH, 4-NO_2

Figure 2.14 Pyrazole derivatives of thiourea and urea

Benzodioxole, thiazole and pyrazole motifs are found in a large number of anticancer drugs and hence to explore their predictable synergistic effect Benzodioxole were combined with thiazolyl-pyrazoline derivatives. Compounds exhibited potential to inhibit HER-2 and had anticancer activities, with compound **45** shown in figure 2.15 being most potent (Wang, Qiu *et al.*, 2013).



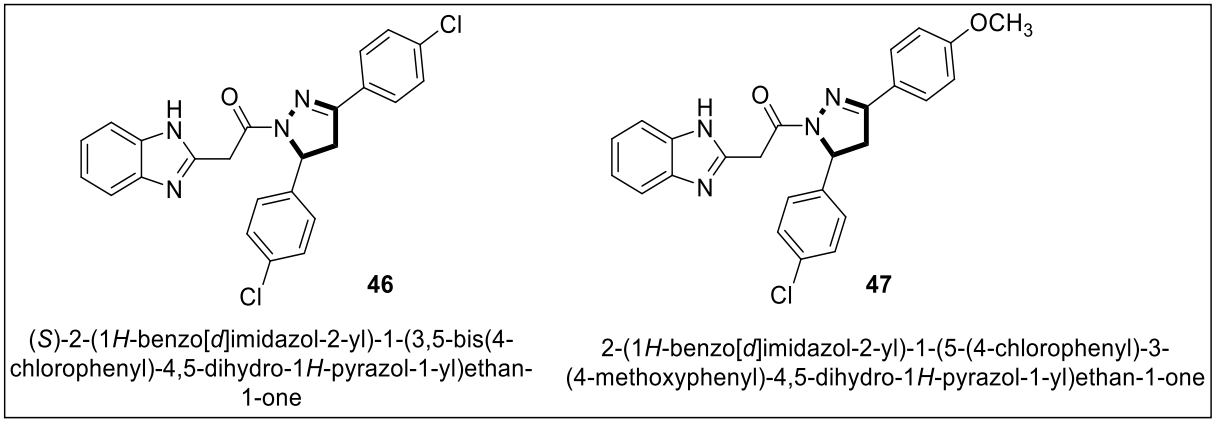
44

45

(*S*)-2-(5-(benzo[*d*][1,3]dioxol-5-yl)-3-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)-4-phenylthiazole compound with bromomethane (1:2)

Figure 2.15 Benzodioxol-thiazol-pyrazole derivatives

Benzimidazole containing pyrazole derivatives exhibit antiproliferative activity, with **47** shown in figure 2.16 being most effective against lung cancer cell lines and in EGFR binding(Akhtar, Khan *et al.*, 2018) .



46

47

(*S*)-2-(1*H*-benzo[*d*]imidazol-2-yl)-1-(3,5-bis(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethan-1-one

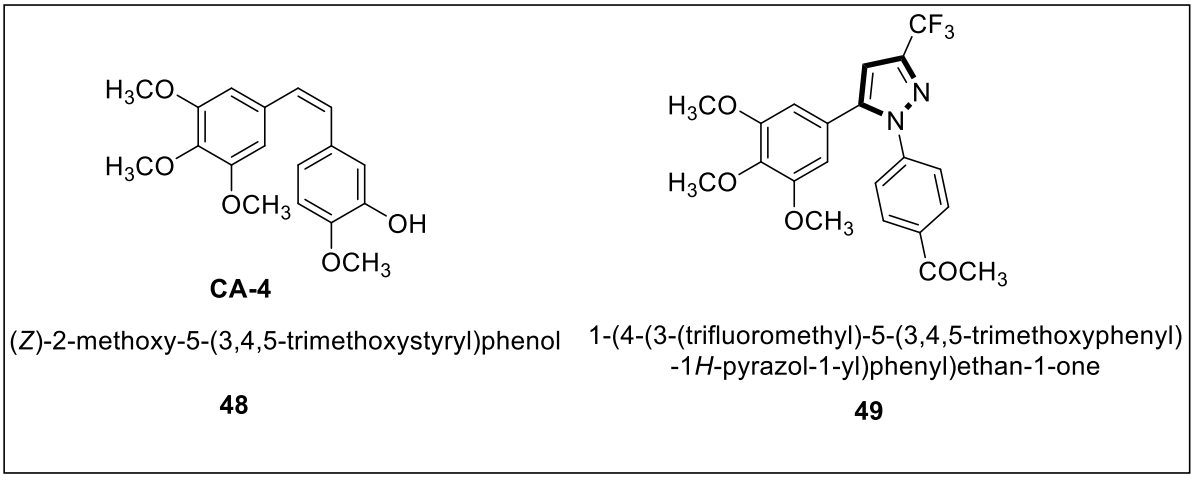
2-(1*H*-benzo[*d*]imidazol-2-yl)-1-(5-(4-chlorophenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)ethan-1-one

Figure 2.16 Benzimidazole containing pyrazole derivatives

2.2.7 Pyrazole as Tubulin Targeting Agents

Dynamic polymers called microtubules form the structural framework of eukaryotic cells. They guide the sister chromatids to the equatorial plane. Any change in dynamics of microtubules alters chromosome separation and halts mitosis leading to apoptosis, making its inhibition a compelling approach for cancer treatment. Tumor suppressor protein p53 is responsible for cell growth, arrest and apoptosis in response to DNA damage or stress and hence called the guardian of genome. p53 mutations are found in around 50% of human cancers.

Some antitubulin agents along with antimitotic activity posses' vascular disrupting and antiangiogenic properties, and can disrupt the existing tumor. One such agent is combretastain A-4 (CA-4) which is a stilbenoid phenol and induces microtubule depolymeriztion on binding. Owing to is poor solubility in water, many derivatives have been synthesized and tested for their anticancer action. Along the same line trifluoromethyl substitution for its pharmacological and physiochemical properties and pyrazole motif which might interact with tubulin with additional hydrogen bond acceptors and donors were incorporated in CA-4 derivatives shown in figure 2.17. Compound **49** was most potent antiproliferative and was found to arrest cells at the G2/M phase of cell cycle (Hura, Naaz *et al.*, 2018).



CA-4

(*Z*)-2-methoxy-5-(3,4,5-trimethoxystyryl)phenol

48

1-(4-(3-(trifluoromethyl)-5-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-1-yl)phenyl)ethan-1-one

49

Figure 2.17 Stilbenoid phenol-pyrazole derivatives

Also p53 on binding to microtubules uses MT-dependent motor complex for targeting nucleus. Inhibition of polymerization due to drugs hinders p53 translocation to the nucleus and hence inhibits activation of downstream targets. p53 activation leading to activation of pro-apoptotic proteins is a recently exploited strategy for drug design. In this regard (Z)-33(2,8-dihydroindeno[2,1-c]pyrazol-3-yl)methylene)indolin-2-one analogues were synthesized and were found to exhibit cytotoxicity and metaphase arrest. Compounds **50, 51** and **52** shown in figure 2.18 exhibited highest activity(Khan, Garikapati *et al.*, 2018).

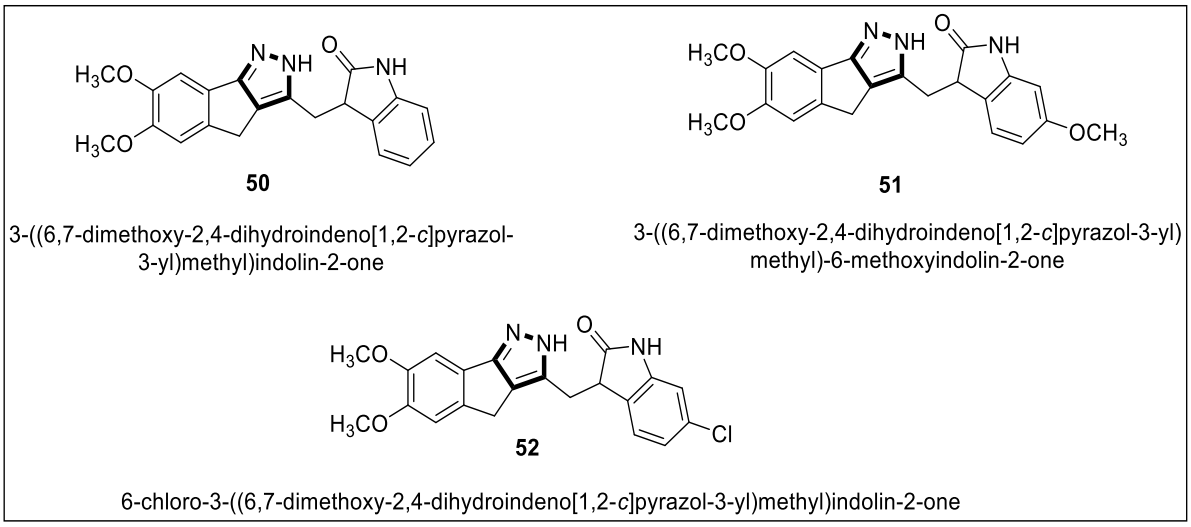
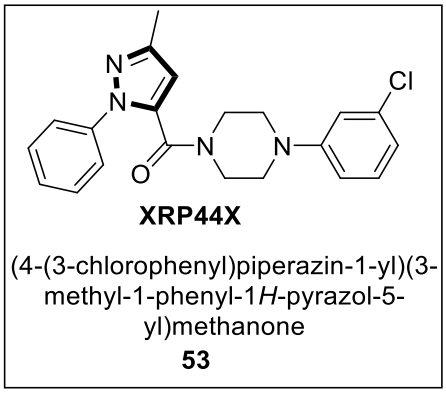


Figure 2.18 Dihydroindeno-pyrazol-methylene Indolinone analogues

2.2.8 Pyrazole as Ras-Net Pathway Inhibitors

Ras-extracellular-signal-regulated kinase (Erk) activates Net, which is a transcription factor; on phosphorylation. It is involved in wound healing, angiogenesis and tumor growth. Pyrazole XRP44X shown in figure 2.19 has been reported to inhibit fibroblast factor 2(FGF-2) induced Net phosphorylation. Further it binds to colchicines-binding site of tubulin, depolymerizes microtubules, stimulates cell membrane blebbing and affects morphology of actin skeleton. This differs from other microtubule targeting agents due to its effect on the Ras-Net pathway, in addition to inhibition of cell growth, cell cycle progression, and aortal sprouting (Wasylyk, Zheng *et al.*, 2008).



XRP44X

(4-(3-chlorophenyl)piperazin-1-yl)(3-methyl-1-phenyl-1*H*-pyrazol-5-yl)methanone

53

Figure 2.19 Pyrazole inhibiting Ras-Net pathway

2.2.9 Pyrazole as Topoisomerase Inhibitors

During the process of replication, transcription, recombination, repair, and chromatin remodeling the DNA strands get entangled and Topoisomerases are nature's solution to this problem (Chen, Chan *et al.*, 2013). Topoisomerase cleave the DNA strands transiently and then relegate it by transestrification reaction, via formation of covalent bond between DNA and the enzyme and hence relaxing the super coiled DNA. Tyrosine is the active site for the reaction. It is these cleavage reaction intermediates which are the targets for various cytotoxic agents. Since topoisomerases have significant roles in many cellular processes, they have been one of the favorite anticancer targets. Drugs like ETP[3] and CPT (2-4) stabilize the covalent complex between DNA and enzyme and hence hinder its catalytic activity. There are two types of topoisomerases topo I and topo II. Topo II cleaves both the strands of the DNA while topo I cleaves only one strand as shown in figure 2.20 (Jacob, Aguado *et al.*, 2001).

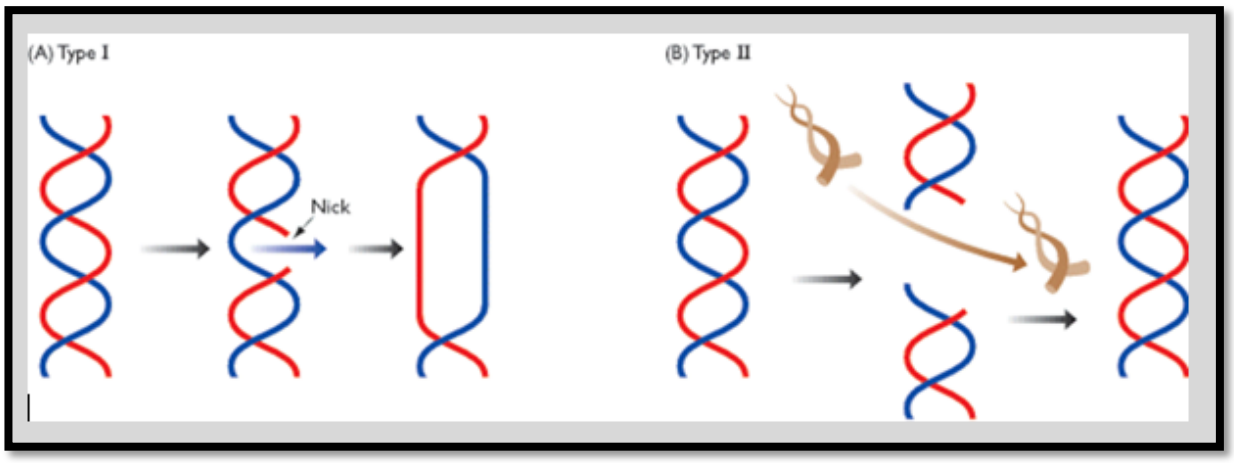


Figure 2.20 Mechanism of action of topo 1 and topo 2

The human topo or the htopo II, further has two subclasses, htopo IIα and htopo IIβ. They are isozymes which have similar catalytic site but differ in their biological functioning. While topo IIα is associated with cell division, topo IIβ is involved in differentiation of the cells (Ganapathi andGanapathi, 2013).

Inhibition of topo can be attributed to restraining of the ligation step during the cell cycle progression. It generates single and double stranded breaks that harm the integrity of the genome and hence induce apoptosis. Thus the topo inhibitors are designed as such, so as to interfere with the action of topo enzymes involved in the breaking and religating of the phosphodiester backbone of DNA strands throughout the regular cell cycle. Topo I inhibitors include Amscarine, irinotecan, topotecan, fluoroquinolones and Camptothecin. There are two classes of drugs which target topo II. First one leads to higher levels of DNA covalent complexes, like etoposide, doxorubicin, and mitoxantrone shown in figure 2.21. Since they involve DNA strand breaks and proteins bound to DNA these are called topo II poisons. While the second class exhibit their cytotoxicity by inhibiting the enzymatic activity and hence called topo II catalytic inhibitors (Nitiss, 2009).

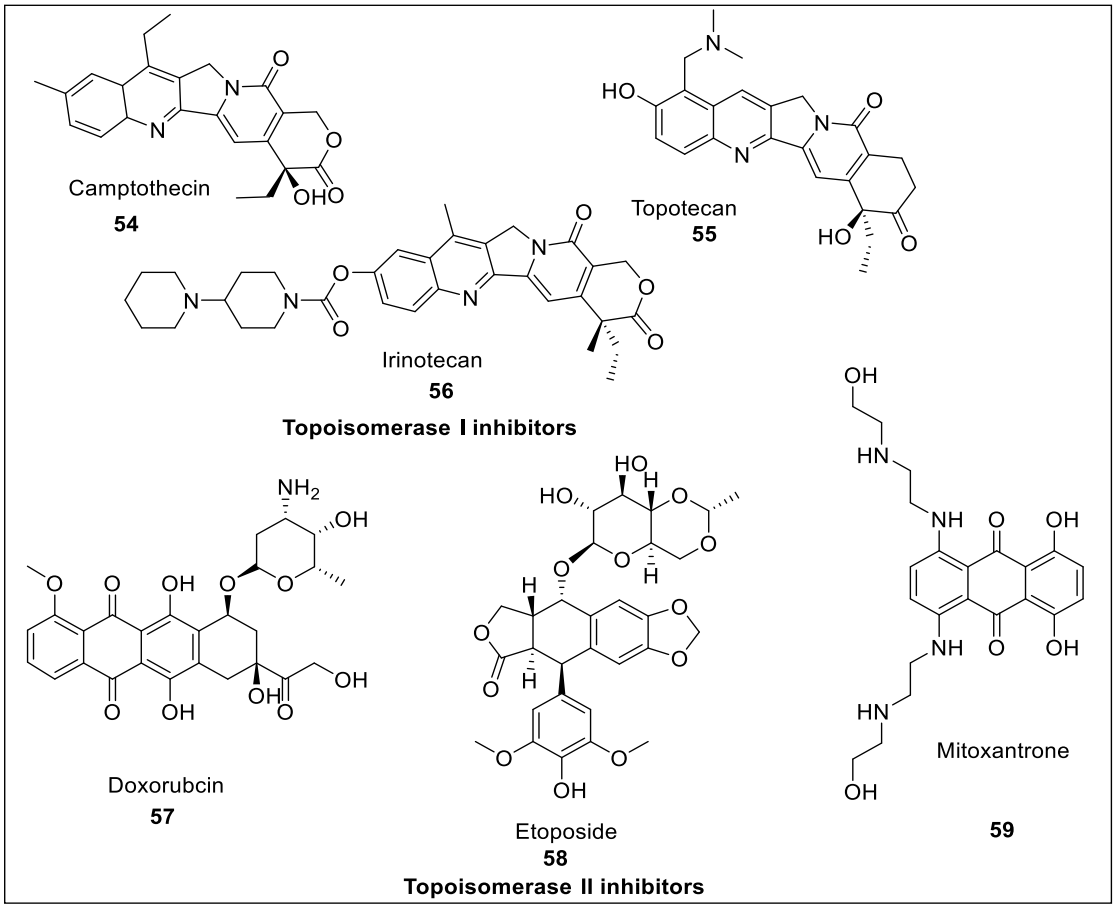


Figure 2.21 Topoisomerase Inhibitors

Teloxantrone HCl and Iosoxantrone are topoisomerase II inhibitors which contain pyrazole moiety and are under clinical trial, shown in figure 2.22. Teloxantrone HCl is an anthrapyrazole antineoplastic antibiotic, which upon introduction to DNA interacts with topoisomerase II. It hinders DNA replication and repair. It also curbs RNA and protein synthesis Similar to teloxantrone, Iosoxantrone is also an anthrapyrazole based antineoplastic antibiotic, which intercalates with DNA. It induces single and double stranded DNA breaks and hence inhibits topo II, leading to inhibition of DNA replication and repair. It also prevents RNA and protein synthesis. Further it is less cardio toxic than doxorubicin (Begleiter, Lin *et al.*, 2006).

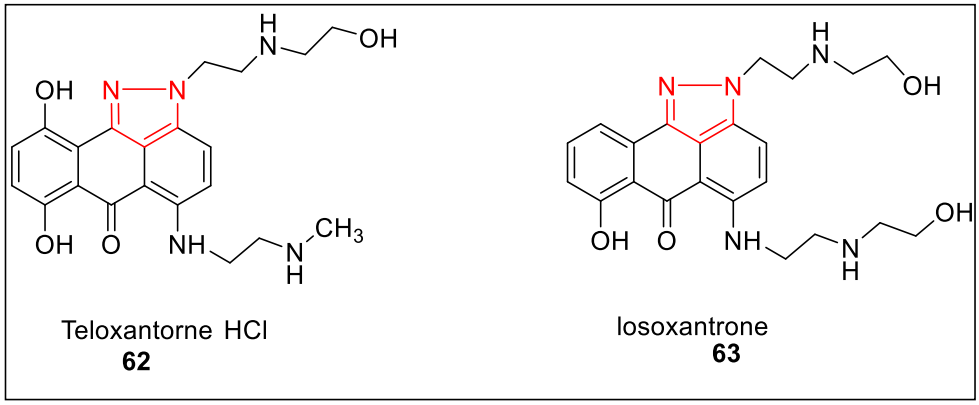


Figure 2.22 Topoisomerase II inhibitors having pyrazole moiety

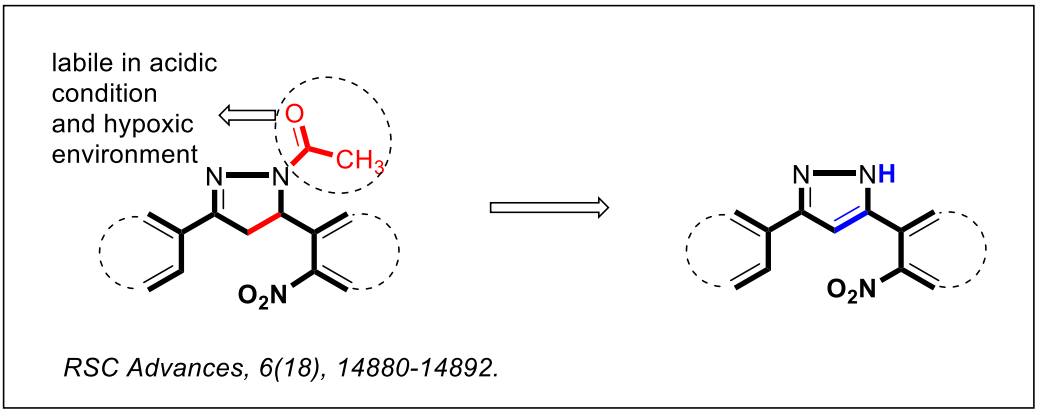
Chapter-3

Rationale

3. Rationale

With the number of targets, types of cancer and resistance mechanisms among the infected cells growing by each day, it is important to develop drug molecules with greater efficacy and lesser toxicity. These drugs must be target specific and effective against the cells which are resistant to the earlier reported and in use drugs.

On reviewing the previous work on anticancer drug synthesis, it can be inferred that pyrazoles based moieties have been considered for designing topo inhibitors. Development of new scaffold or modifications of the existing ones can be justified, if it leads to greater affinity towards the target or forms a more potent and stable form.



RSC Advances, 6(18), 14880-14892.

In the present work we have synthesized pyrazole based moiety.

Pyrazoline based compounds having an acetyl group are reported as topo inhibitors. . We in our research work have attempted to synthesize pyrazole based moieties devoid of an acetyl group which is liable in acidic environment of cancer cells.

We attempt to synthesize more stable pyrazoles in comparison to pyrazolines. The stability can be attributed to the presence of one extra endocyclic double bond than their corresponding pyrazolines.

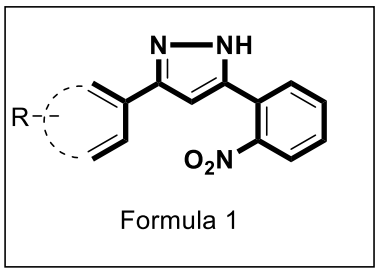
Chapter- 4

Objectives

4. Objectives

Based on the Rationality we have set following objectives

A. To synthesize pyrazole based heterocycles pertaining to formula 1.



Formula 1

B. To assess *in vitro* antiproliferative activity of synthesized compounds using MTT assay.
C. To perform *in silico* study of the synthesized compounds by docking into topoisomerase 1 and 2 enzymes.

Chapter-5

Material and Methods

5. Material and Methods

5.1 General: Synthesis

1. All the reagents and solvents were purchased from Sigma-Aldrich, Loba-Chemie Pvt. Ltd., Avra Synthesis (AR/GR quality) were used without any additional purification.
2. For weighing purposes Sartorius Analytical balance (BSA224S-CW) was used.
3. ILMVAC Rotary evaporators were used for evaporating solvents and JSGW Heating mantle for reflux reactions.
4. The progress of reaction was monitored by TLC, petroleum ether/ethyl acetate or chloroform/methanol used as mobile phase on pre-coated Merck TLC plates (in JSGW UV/fluorescent analysis cabinet or iodine chamber).
5. Melting points were recorded on Stuart melting point apparatus (SMP-30) with open glass capillary tubes and were uncorrected.
6. The Compounds were further purified using flash chromatography (Biotage) or column chromatography.
7. The Mass (EI) spectra of compounds were recorded on Shimazdu *GCMS-QP2010* at Central Instrumental Laboratory (CIL), Central University of Punjab.
8. Infrared (IR) spectra of compounds were recorded with KBr/neat on a Bruker FT-IR spectrophotometer at Central Instrumental Laboratory (CIL), Central University of Punjab.
9. ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra was recorded at Central University of Rajasthan and IIT(JEOL ECS), Guru Jambheshwar University of Science and Technology(Avanu III), Hisar in $CDCl_3$/ d_6-DMSO on a Bruker Advance II 400 MHz and 100 MHz respectively using TMS ($\delta = 0$) as an internal standard.

5.2.1 General procedure for the synthesis of chalcones

A substituted aldehyde (300 mg) and a substituted ketone were added (1 equivalent), with methanol (10 mL) as a solvent. 20% sodium hydroxide was used as the base. The reaction mixture was stirred for 4 hours at room temperature. On completion of the reaction (confirmed using TLC), the reaction mixture was poured in water. The mixture was filtered. The residue obtained was further washed with water and dried to obtain the crude product. Recrystallization was done in methanol to obtain pure compound. The synthesized chalcones have been enlisted in figure 5.1.

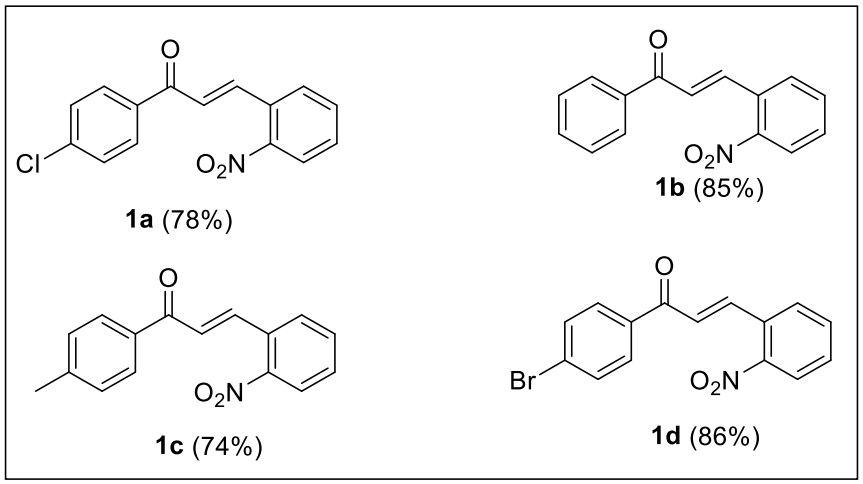


Figure 5.1 Synthesized chalcones **1a-1d**

5.2.2 Step 2: General procedure for the synthesis of Pyrazoline

Chalcone (300 mg) and hydrazine hydrate 80% (3 mL) were stirred in methanol for 5 h. On completion of the reaction (confirmed using TLC), the products were procured by filtering off the excess methanol. Pyrazolines thus obtained was used for next step without any further purification. The synthesized pyrazolines have been enlisted in figure 5.2

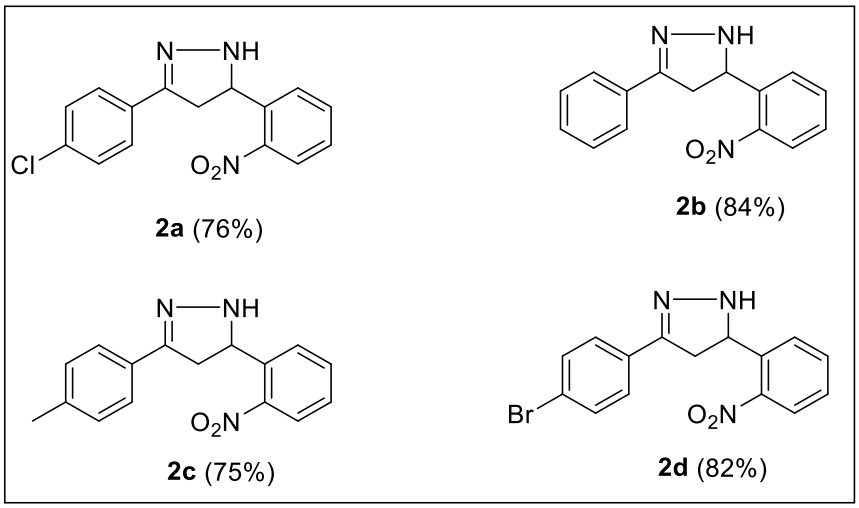


Figure 5.2 Synthesized Pyrazolines **2a-2d**

5.2.3 Step 3: General procedure for the synthesis of pyrazoles.

Pyrazoline (300 mg) along with catalytic amount of molecular iodine in DMSO was refluxed at 60-80 ^{0}C for 4 h. On completion of reaction (confirmed using TLC), the reaction mixture was poured in ice cold water, and the product was extracted using ethyl acetate. Sodium thiosulphate was used to wash the organic layer to clear away traces of iodine, if any. Further the organic layer was passed over sodium sulphate and filtered to eliminate any moisture. Finally the filtrate was evaporated under reduced pressure using a rotary evaporator to obtain the final product. The synthesized pyrazoles have been enlisted in figure 5.3.

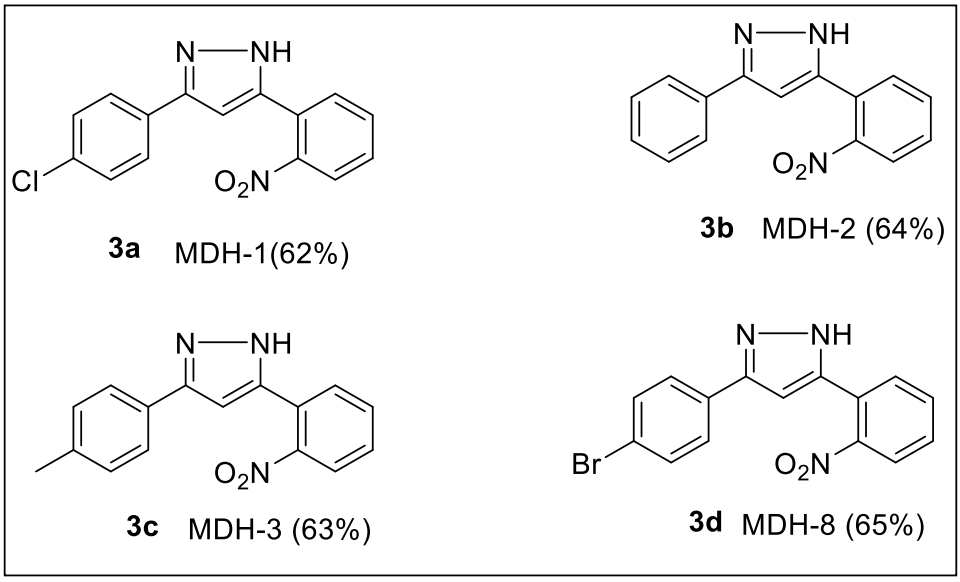
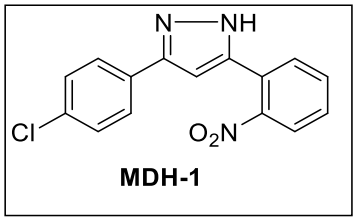


Figure 5.3 Synthesized pyrazoles **3a-3d**

5.2.3.1 Synthesis of 3-(4-chlorophenyl)-5-(2-nitrophenyl)-*1H*-pyrazole (3a)



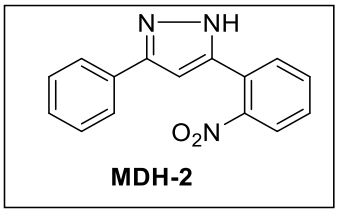
MDH-1

Yield: 62%, brown solid

m.p:136-138 °C.

Mass (EI): m/z: 299

5.2.3.2 Synthesis of 3-phenyl-5-(2-nitrophenyl)-*1H*-pyrazole (3b)



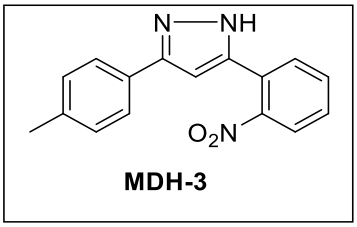
MDH-2

Yield: 64%, orange-brown solid

m.p: 196-198°C.

Mass (EI): m/z: 265

5.2.3.1 Synthesis of 3-(4-methylphenyl)-5-(2-nitrophenyl)-*1H*-pyrazole (3c)



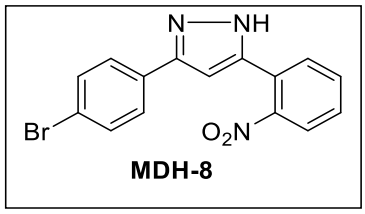
MDH-3

Yield: 63%, dark brown

m.p: 126-128°C.

Mass (EI): m/z: 279

5.2.3.1 Synthesis of 3-(4-bromophenyl)-5-(2-nitrophenyl)-*1H*-pyrazole (3d)



MDH-8

Yield: 65%, yellowish brown solid

m.p: 138-140°C.

Mass (EI): m/z: 343

^{1}H NMR (400 MHz, $CDCl_3$, TMS=0) δ: 8.04 (1H, d, *J*=8Hz), 7.92 (1H, d, *J*=8Hz), 7.79-7.77 (1H, m), 7.69 (1H, d, *J*=7.2Hz), 7.63-7.62 (1H, m), 7.56 (2H, t, J_1=8Hz, J_2=8Hz), 7.49-7.48 (1H, m), 6.66 (1H, s)

^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ: 148.89, 132.23, 132.10, 132.07, 131.86, 131.01, 130.36, 129.64, 129.19, 127.22, 123.92, 122.65, 103.01

UV (methanol): λ_{max} at 230 nm and 298 nm

5.3 Biology

5.3.1 Chemicals

- Cancer cell lines were cultured with media – DMEM, Penicillin/Streptomycin antibiotic solution and Phosphate buffer saline, fetal bovine serum were purchased from Hi Media.
- **MTT** dye used for MTT assay was purchased from Hi Media
- **DMSO,** extra pure AR was purchased from SRL

5.3.2 Instruments

- All experiments involving cell culture was carried out under aseptic conditions under laminar air flow. Cells were counted on automated cell counter and incubated in the presence of carbon dioxide in the incubator at 37°C. Finally centrifugation was done and inverted microscope was used to observe cancer cells. The absorption was measured by using spectrophotometer.

Table 5.1 List of Instruments Used in Biological Evaluation

Instruments	**Purpose**	**Company**
Automatic cell counter	For counting of cells	Invitrogen
Incubator	Incubation	Galaxy
Inverted microscope	Visualization of cancer cells	Magnus, Olympus
Laminar air flow	For aseptic conditions	Macro Scientific Works
Centrifuge 5430 R	Centrifugation	Eppendorf

5.3.3 Cell lines under study

MDA-MB 231 breast cancer cell line was used for evaluation of antiproliferative assay.

MDA-MB 231- It is a human breast cancer cell line that was established from pleural effusion of a 51-year-old Caucasian female with a metastatic mammary adenocarcinoma. MDA-MB-231 is a highly aggressive and poorly differentiated triple-negative breast cancer (TNBC) cell line as it lacks estrogen receptor (ER) and progesterone receptor (PR) expression, as well as HER2 (human epidermal growth factor receptor 2) amplification.

5.3.4 Routine assay in culture laboratory

A. Culturing of cell lines

DMEM is used as medium for culturing the cancer cell lines as cell adherent, trypsin was added to remove them from the surface (trypsinization). Cells were harvested in 5 ml media containing serum which inactivates trypsin enzyme. Harvested cells were centrifuged at 1200 rpm at 4°C for 5 minutes, supernatant was removed and pellet was re-suspended in media (2 ml). With automated cell counter, cell number was counted. The cells were moved to fresh media every three days.

B. Maintenance and sub-culturing of cell lines

Maintenance and culturing of cell lines was done in 25 cm^2 or 75 cm^2 flasks having DMEM medium supplemented with 10% fetal bovine serum (FBS), 1X antibiotic solution and incubated at 37° C in a humidified atmosphere containing 5% CO_2.

Further sub-culturing of cells was done in 25 cm^2 flasks until the cancer cell lines have reached 70-80% of growth. The reagents vital for the procedure were placed in water bath maintained at 37° C for 10-15 minutes and trypsin was added for the detachment of adherent cells.1 ml of medium containing

serum was added after 5 minutes to stop the action of trypsin. Then, the cells were transferred to 15 ml centrifugation tubes and centrifuged for 5 min at 1200 rpm at 4° C. The supernatant was removed and the cell pellet was again re-suspended in complete media. The cell lines were transferred to fresh media every two days (cell passaging).

7.2.5 Evaluation of antiproliferative activity of the synthesized compounds (MTT Assay)

MTT is an *in vitro* colorimetric assay used for the measurement of cell proliferation (Mosmann, 1983 #87). It is also known as cell viability test. The tetrazolium compound **MTT** (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) is reduced to an insoluble purple colored formazan product by mitochondrial reductase or succinate dehydrogenase in metabolically active cells only as shown in figure 5.4. When formazan passes to the mitochondria it gets solubilized with DMSO and can be measured spectrophotometrically.

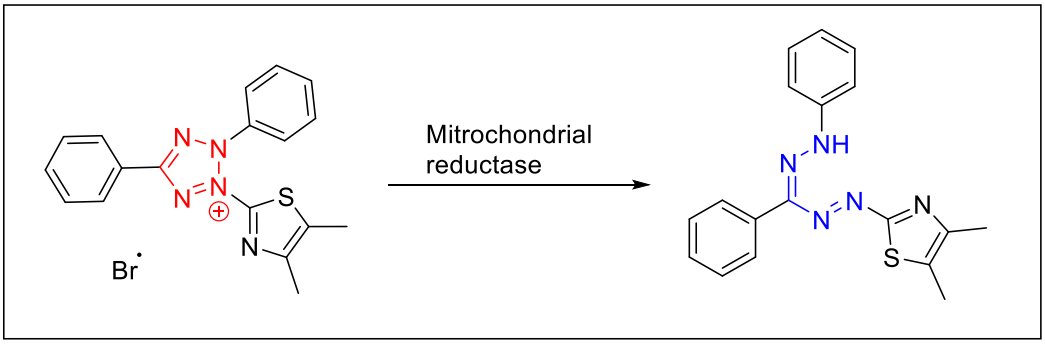


Figure 5.4 Reduction of MTT

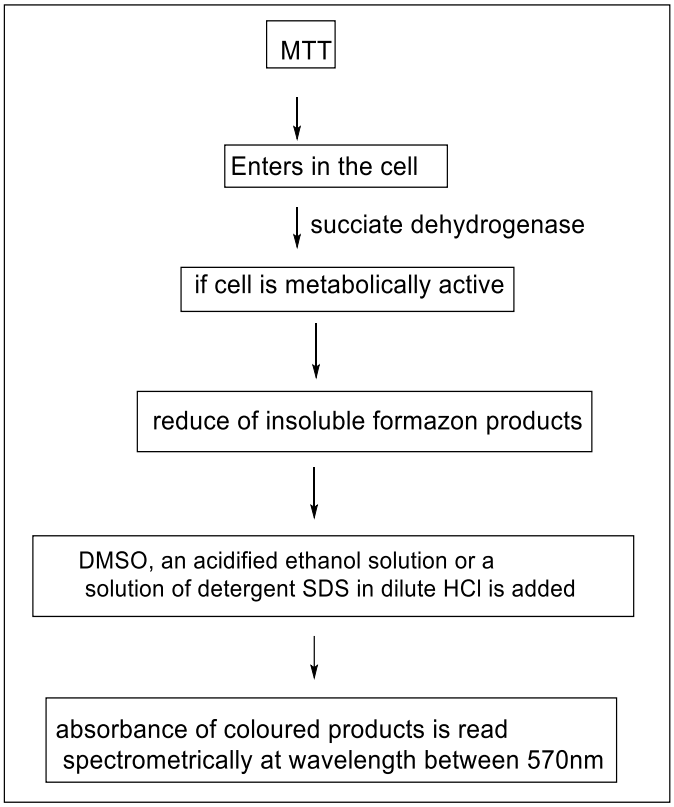


Figure 5.5 Basic Principle of MTT Assay

Material: MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), Phosphate buffer solution, DMSO (Dimethyl sulfoxide)

Procedure: The cells from the cancer cell line- MDA-MB-231 were counted on the automated cell counter. Nearly the 8,000-10,000 cells were seeded in each well of the 96 well plates. The plate was incubated at 37° C with 5% CO_2 for 24h followed by serum starvation for 8 h for synchronization and replenishing with complete media. The treatment was given to the cells in triplicate concentration of 1 µM, 5 µM, and 25 µM and incubated for 48h. The MTT solution (5mg/10mL) was added after removing the media from each well and incubated in the dark for 4 h., the MTT solution was removed from each well and the intracellular precipitate was dissolved in DMSO solution and the absorbance of the dark violet color formed as consequence of DMSO addition is read spectrometrically at 570 nm which was expressed as % inhibition (Mean ± S.D)

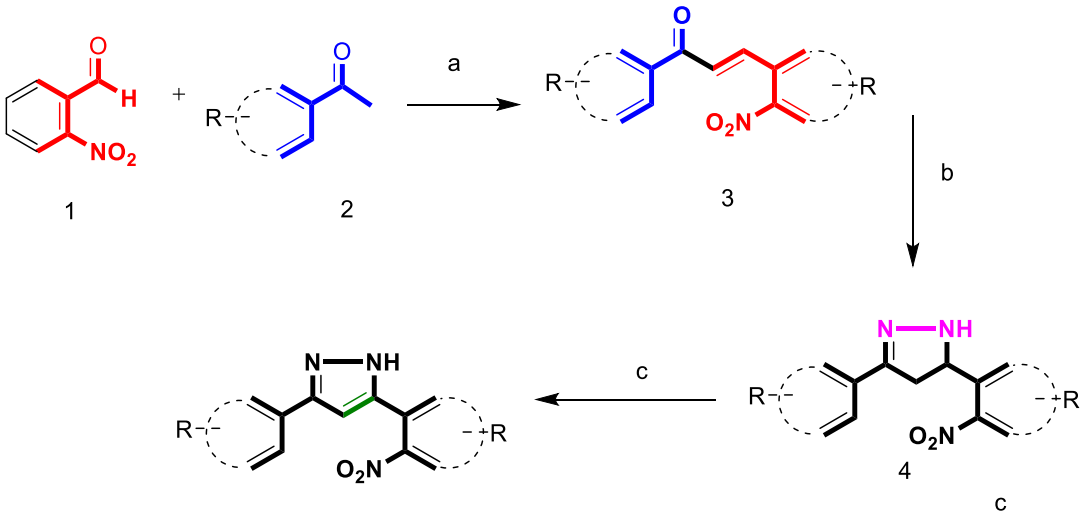
Chapter-6

Results and Discussion

6. Result and discussion

6.1 Synthesis

We broach the route shown in scheme 6.1 for the synthesis of the desired compounds.

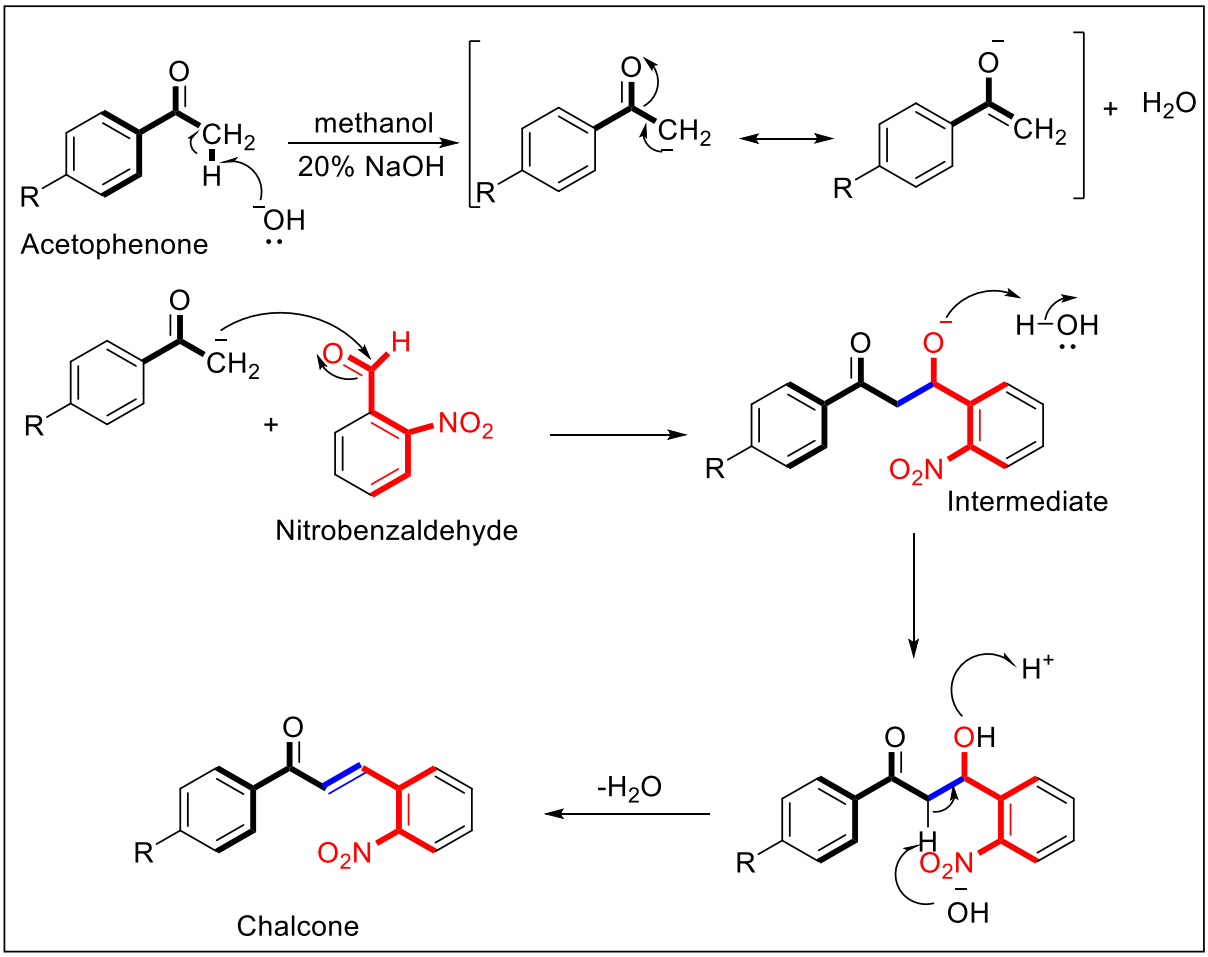


Reagents and conditions: (a) 20%, NaOH,Methanol, 4-5h (b)hydrazine hydrate, MeOH,rt (c) I_2 ,DMSO,reflux,4h

Scheme 6.1 Proposed route for the synthesis of target compounds

6.1.1 Synthesis of chalcones

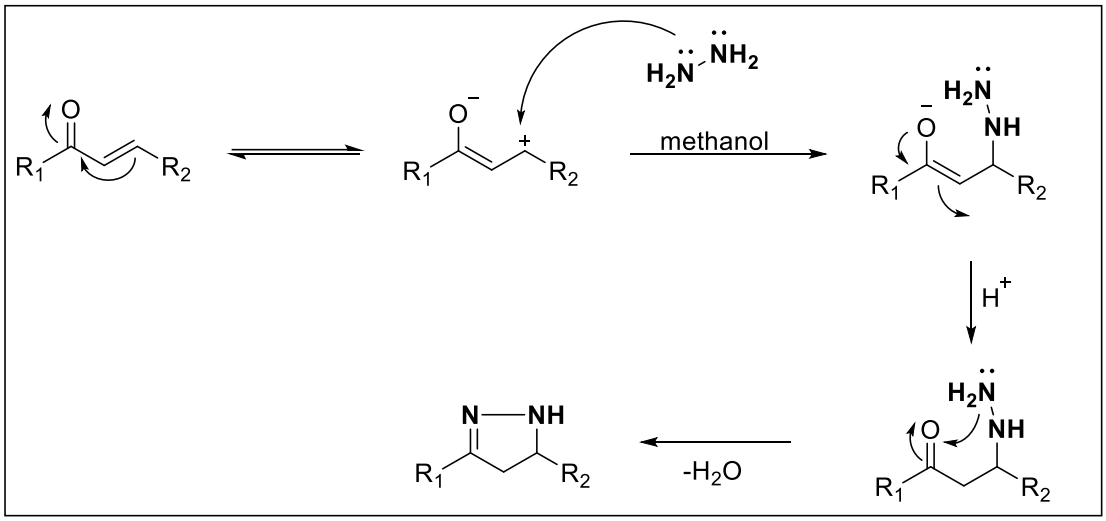
Chalcone derivatives were synthesized via a base catalyzed Claisen-Schmidt condensation. In the reaction, the base abstracts a proton from the α-carbon which leads to the formation of an enolate ion. This enolate [(nucleophile) carbanion] attacks the carbocationic center of the aldehyde, resulting in a dehydration reaction or removal of an alcohol molecule along with formation of the desired chalcones as shown in scheme 6.2.



Scheme 6.2 Mechanism for Synthesis of Chalcone

6.1.2 Synthesis of Pyrazoline

Pyrazoline synthesis involves Michael Addition reaction, which begins with the attack of lone pair of Nitrogen of hydrazine at the electrophilic center of the resonance stabilized α, β unsaturated ketone i.e. Chalcone. This leads to formation of an enolate intermediate which stabilizes by forming a carbonyl group and addition of proton from solvent at the negatively charged α-carbon. Further a hydride shift from nitrogen and release of water molecule leads to formation of cyclized pyrazoline products as shown in scheme 6.3.

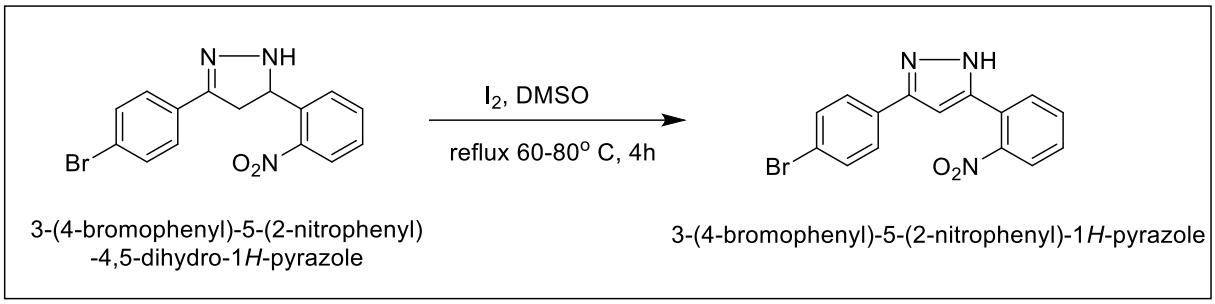


Scheme 6.3 Mechanism for Synthesis of Pyrazoline

6.1.3 Synthesized compounds

The synthesized pyrazolines were oxidized to pyrazoles in presence of catalytic amount of Iodine in DMSO.

Synthesized 3-(4-bromophenyl)-5-(2-nitrophenyl)-1H-pyrazole [**3d** (MDH-8)] was characterized by mass, UV and NMR analysis.



3-(4-bromophenyl)-5-(2-nitrophenyl) -4,5-dihydro-1*H*-pyrazole

3-(4-bromophenyl)-5-(2-nitrophenyl)-1*H*-pyrazole

The mass spectrum showed a peak at m/z 343 as the base peak. A peak is found adjacent to the base peak at m/z 345 indicates the presence of Br in the compound.

The proton NMR spectrum showed a characteristic doublet at 8.04 for single H due to highly deshielded proton adjacent to nitro group. A doublet at 7.92 and multiplet at 7.79 for one H each belong to the protons adjacent to Br. A triplet for two H at 7.56 belongs to the protons on the ring containing NO_2. A singlet at 6.66 is for the proton at the double bonded carbon.

The UV absorbance spectrum in methanol shows two energy bands with λ_{max} at 230 nm and 298nm. The high energy band at shorter wavelength (227-233 nm) is due to the π-π transition while the lower energy band (296-302 nm) represents the n-π transition. A shift in λ_{max} towards longer wavelength (lower energy) has been observed on formation of pyrazole from its respective pyrazoline owing to the increased conjugation and hence a decreased energy for the transition.

The pyrazoles (3a-3d) listed in figure 6.1 were synthesized.

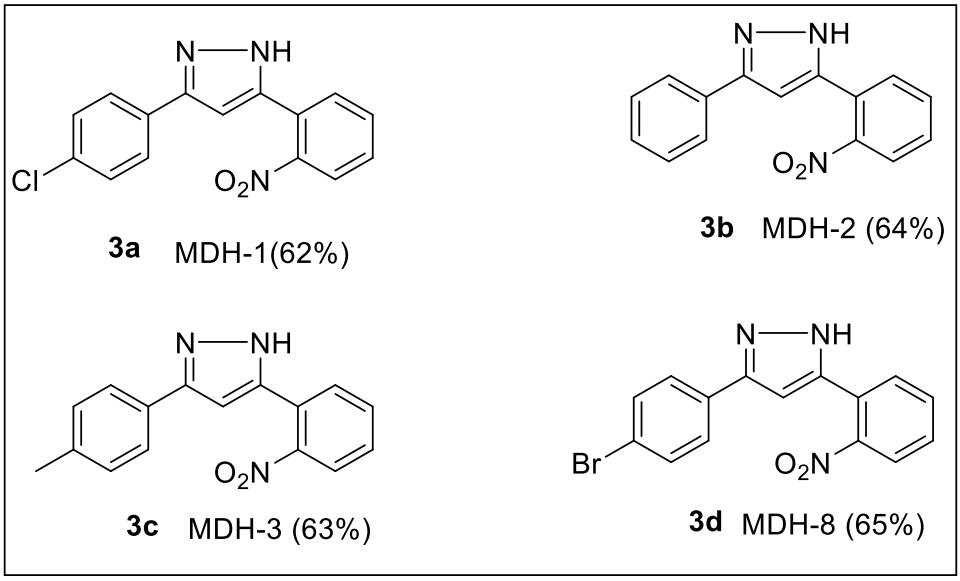


Figure 6.1 Synthesized pyrazoles

6.2 Biological studies

6.2.1 Evaluation of antiproliferative properties of the compounds

For the evaluation the anti-proliferative potential of the synthesized compounds MTT based assay was done by using MDA-MB 231 breast cancer cell lines. Approximately 8,000-10,000 cells (100 µL/well) were seeded in 96 well plates and treated with synthetics at 1, 5 and 25 µM concentration respectively in triplicate for 48 h followed by MTT assay and plotted for % cell survival. The values obtained were expressed as % inhibition (Mean ±S.D.) on Y-axis and were represented graphically with compounds plotted at X-axis. Both the compounds showed good activity against both the cell lines as described below. Further, the IC_{50} value was determined using linear regression method by plotting a trend line (line scattered plot) in excel.

6.2.1.1 Antiproliferative activity against MDA-MB 231 (breast cancer) cell line

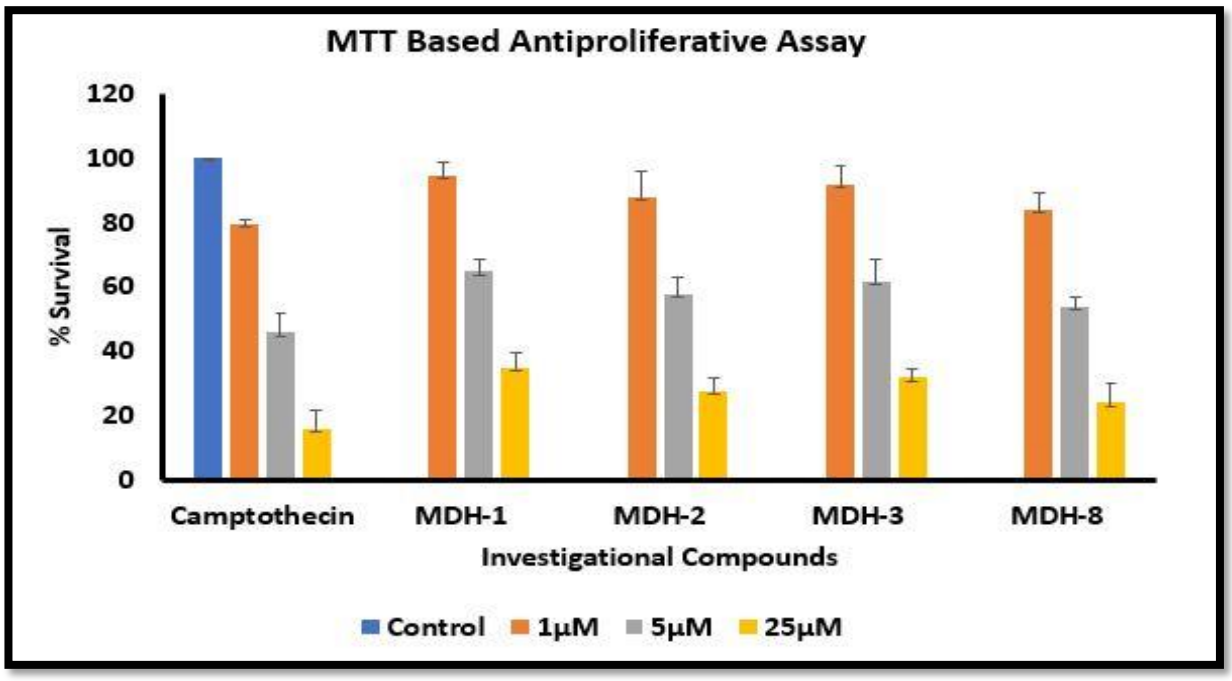


Figure 6.2 Percentage survival of MDA-MB 231 cell line in response to treatment with synthesized compounds at concentrations of 1µM, 5µM and 25µM for 48 hours

The synthesized compounds MDH-1, MDH-2, MDH-3 and MDH-8 were evaluated for the antiproliferative activity.

- Inhibition increases logarithmically with increasing concentration for all the compounds.
- Synthesized compounds inhibit 60-65% cells at a concentration of 25µM.

6.2.1.2 IC_{50} value: IC_{50} can be defined as the inhibitory concentration of the compound at which 50% of the target is inhibited. It measures the effectiveness of the drug in its ability to inhibit a certain biological function.

Table 6.1 IC_{50} Values of the investigational compounds

S. No	**Investigation compound**	**IC_{50} (µM) +SD (6.24)**
1.	Camptothecin	4.74
2.	MDH-1	14.48
3.	MDH-2	10.16
4.	MDH-3	12.41
5.	MDH-8	7.68

6.3 Molecular modeling studies

The molecular modeling studies show the interaction between small molecules (ligands) and macromolecules target (receptor) and their binding capacity. The compound MHD-8 with IC_{50} value7.68 docked in the active sites to identify the interaction with topo I and II. The suitable interaction between ligand and receptor were scored (module of Schrödinger Suite).

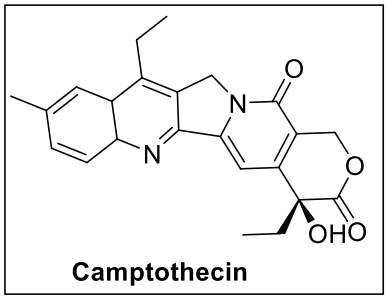
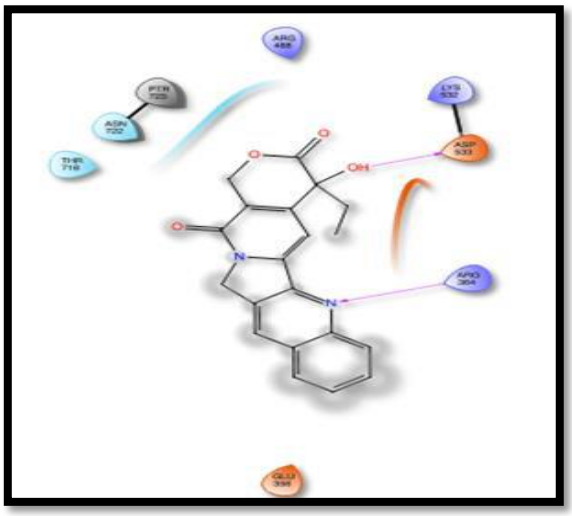
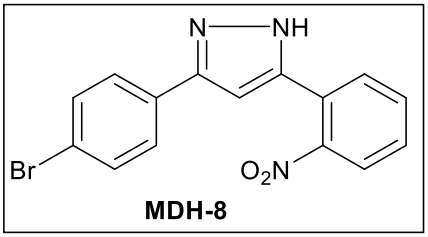
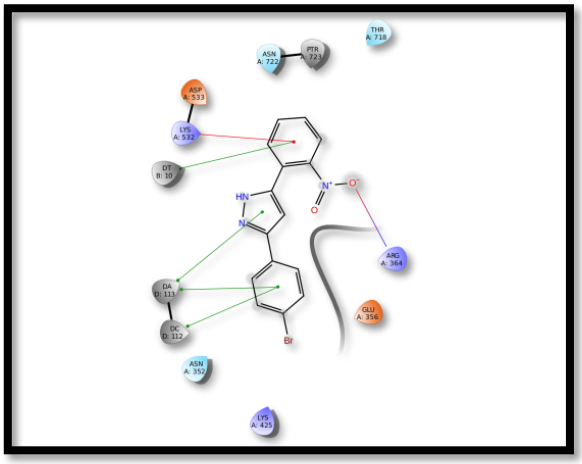
Table 6.2 Docking Score of Pyrazole MDH-8 with 1T8I for Interaction with Topoisomerase 1

TOPOISOMERASE 1 (1T8I)		
S. No	**CODE**	**DOCK SCORE (kcal/mol)**
1.	CAMPTOTHECIN	-8.829
2.	MDH-8	-5.119

Table 6.3 Docking Score of Pyrazole MDH-8 with 1ZXM for Interaction with Topoisomerase 2

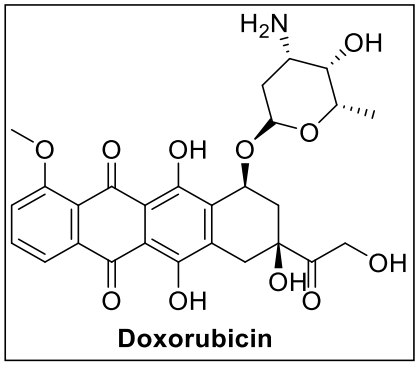
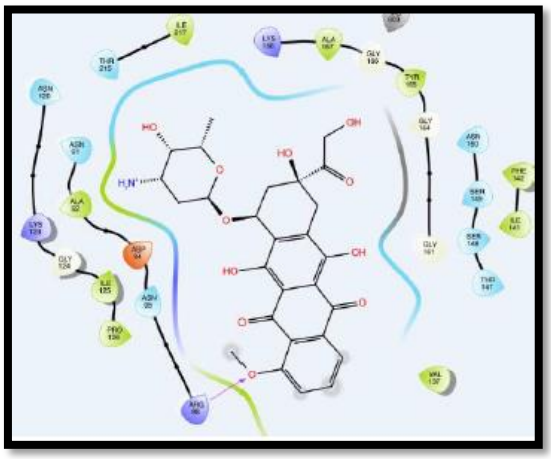
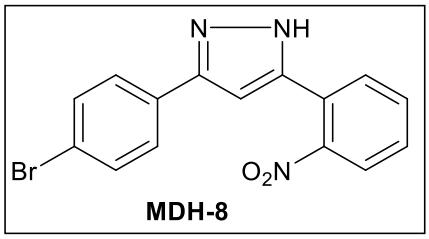
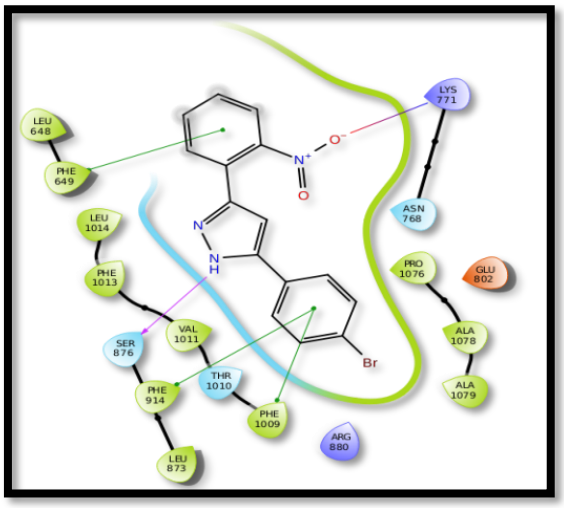
TOPOISOMERASE 2 (1ZXM)		
S. No	**CODE**	**DOCK SCORE (kcal/mol)**
1.	DOXORUBICIN	-4.641
2.	MDH-8	-5.32

Table 6.4 Docking study of Camptothecin and MDH-8 for their interaction with Topo 1

TOPOISOMERASE 1		
Structure (Name)	**Docking pose**	**Dock score**
 Camptothecin		-8.829 kcal/mol
 MDH-8		-5.119kcal/mol

Interactions for Camptothecin include two H bonds which are formed between the hydroxyl group and aspartic acid and the other one is between arginine and pyridine ring. Pyrazole MDH-8 shows a π-cation interaction between lysine and the phenyl ring containing nitro group. It also forms a salt bridge with the nitro group. Additionally, 4 π-π stacking interactions with unknown residues were also observed.

Table 6.5 Docking study of Doxorubicin and MDH-8 for their interaction with Topo 2

TOPOISOMERASE 2		
Structure (Name)	**Docking pose**	**Dock score**
 Doxorubicin		-4.641 kcal/mol
 MDH-8		-5.32 kcal/mol

Methoxy oxygen of Doxorubicin forms an H bond with arginine while for MDH-8 same is formed between the N of the pyrazole ring and serine. Three π-π stacking interactions occur between the phenyl rings and phenylalanine. A salt bridge is also formed between the nitro group and lysine.

Chapter 7

Conclusion

7. Conclusion

Over the years, with the evolution of mankind and its life style the number and types of cancer has been on a rise. With each passing day a new facet about cancer, its targets, their inhibition, resistance towards drugs comes to our knowledge and hence making it further more important to design and synthesize drugs with greater efficacy and lesser or no side effects.

Keeping this in mind the newly synthesized compounds having stable pyrazole scaffold were evaluated and explored for their antiproliferative activity on breast cancer cell line (MDA-MB 231) by performing MTT assay. The compound MDH-8 has an IC_{50} value of 7.68. Compounds MDH-2, MDH-3 and MDH-8 show an inhibition of around 60% at a concentration of 25µM.

Docking scores infer that the synthesized pyrazole MDH-8 shows better interaction with topoisomerase 2 than topoisomerase 1 and can hence be further explored for its potentiality in topoisomerase 2 inhibition.

Results connote that the synthesized compounds can supplement in lead optimization, generation of library compounds and their screening against various cell lines. They can be useful in drawing out structure activity relationships and hence developing a promising drug.

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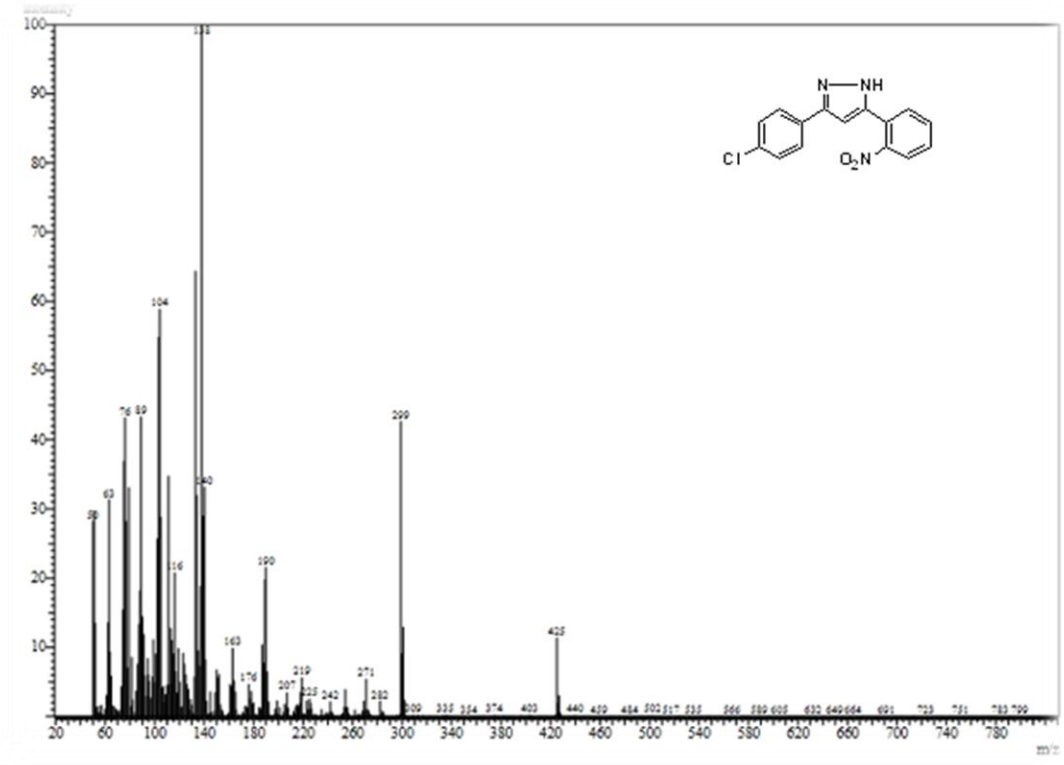
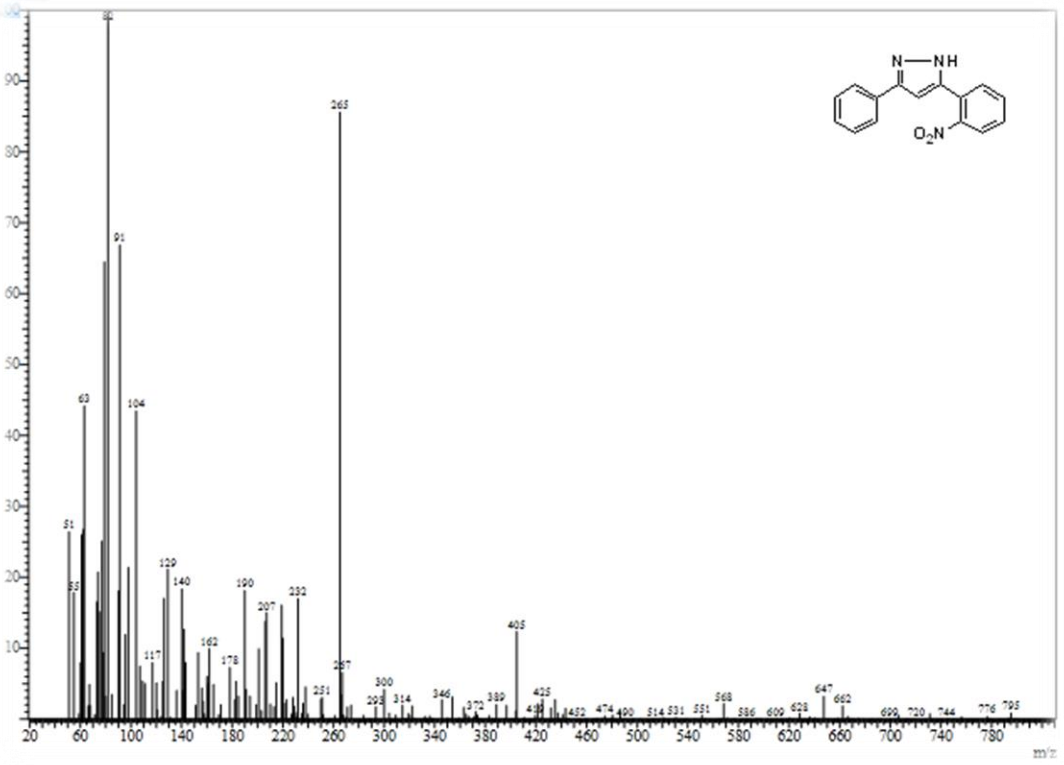
APPENDIX-A
Spectral Data of Representative Compounds



Mass spectra of MDH-1



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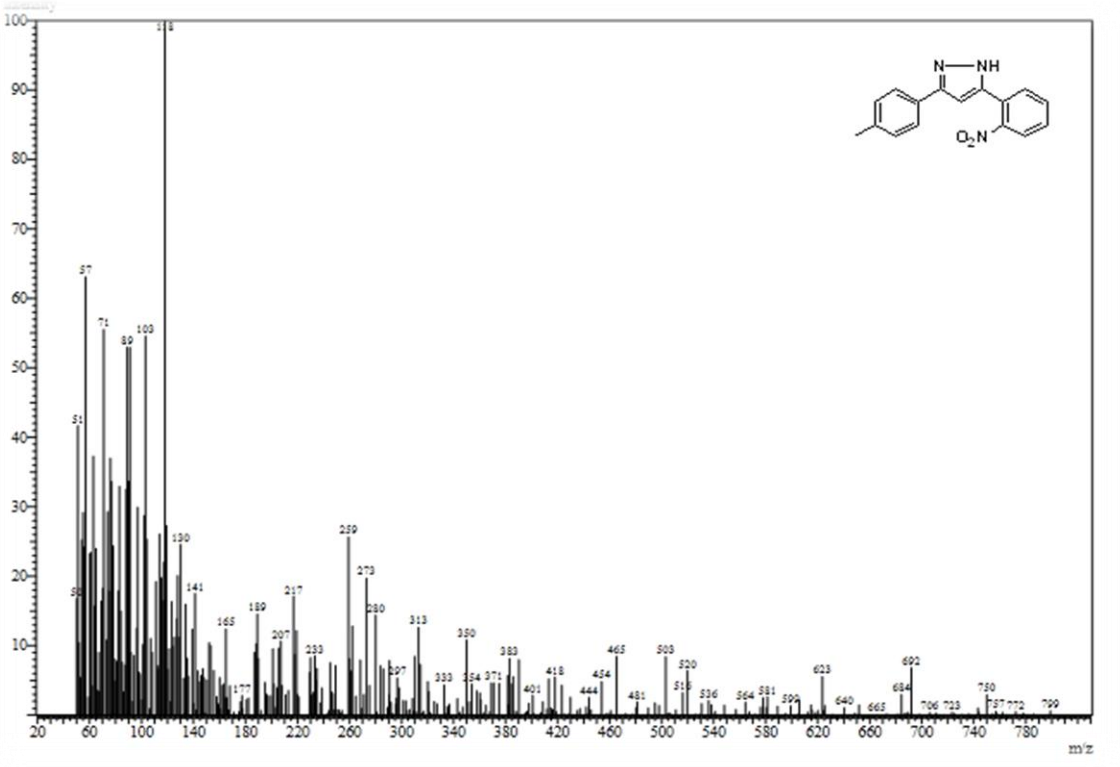

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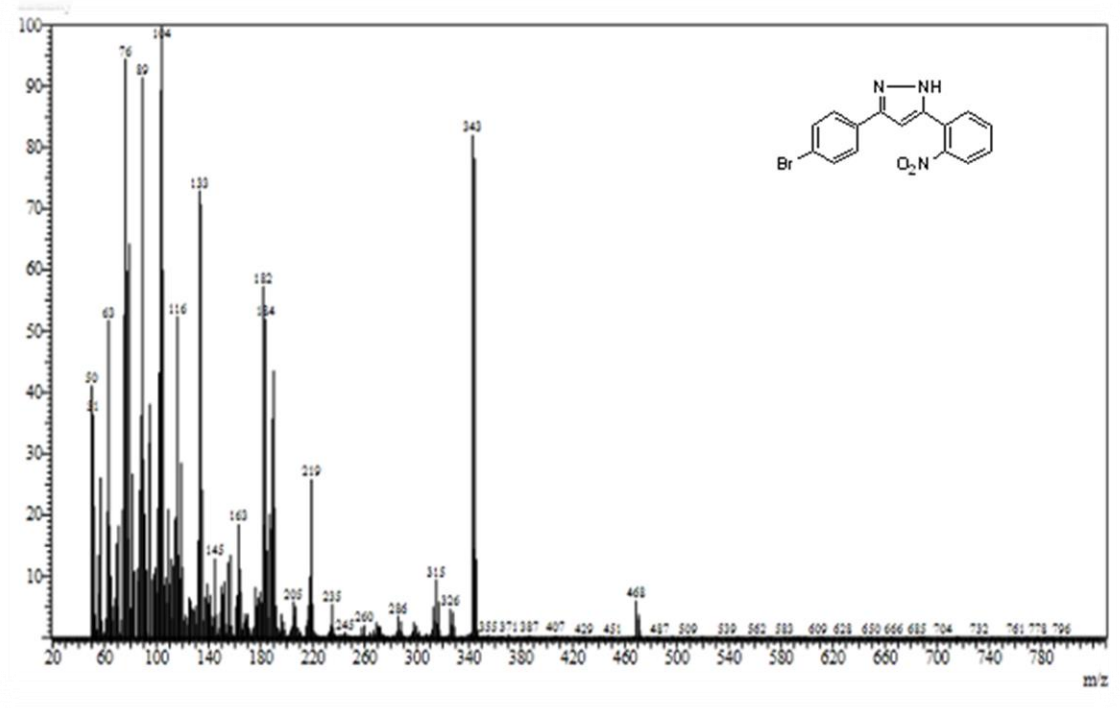
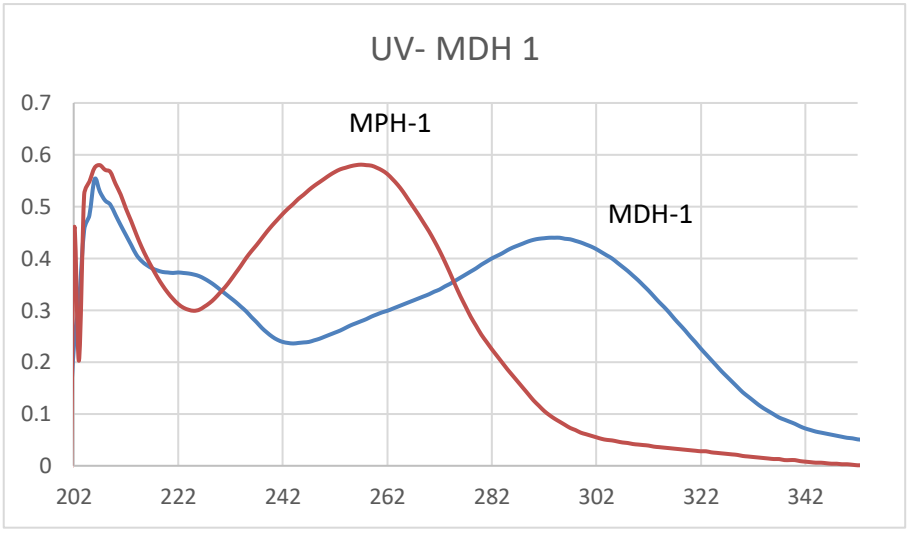
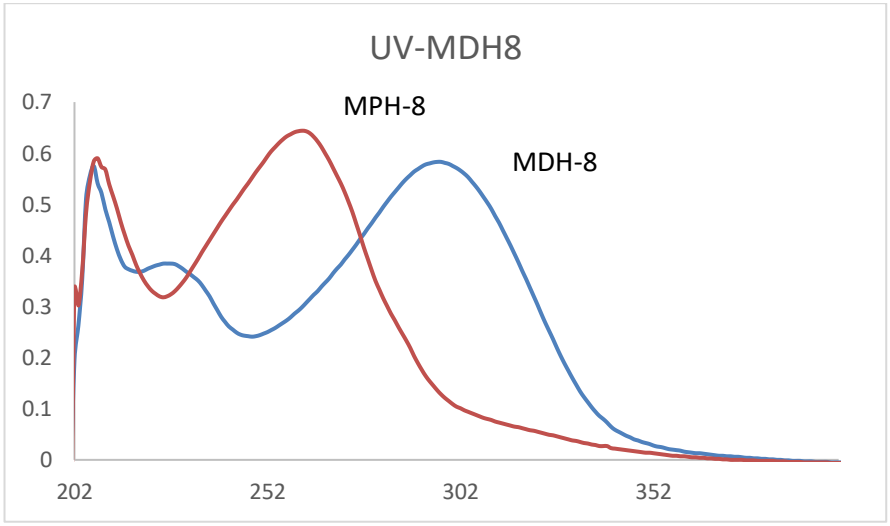


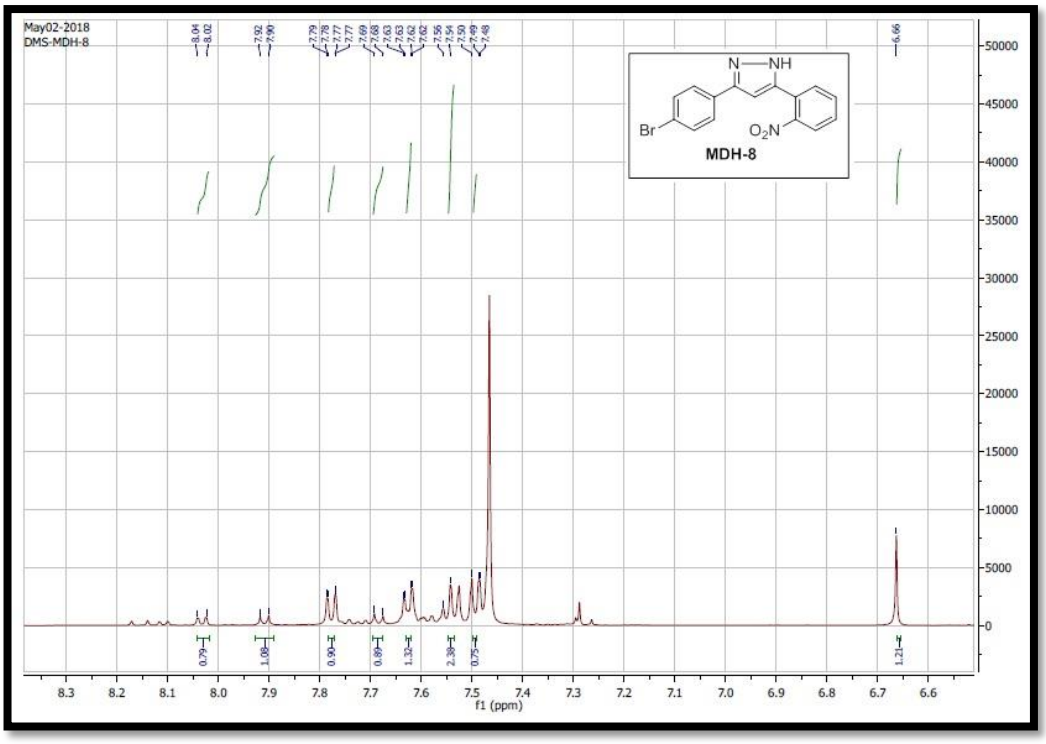
Mass spectra of MDH-8



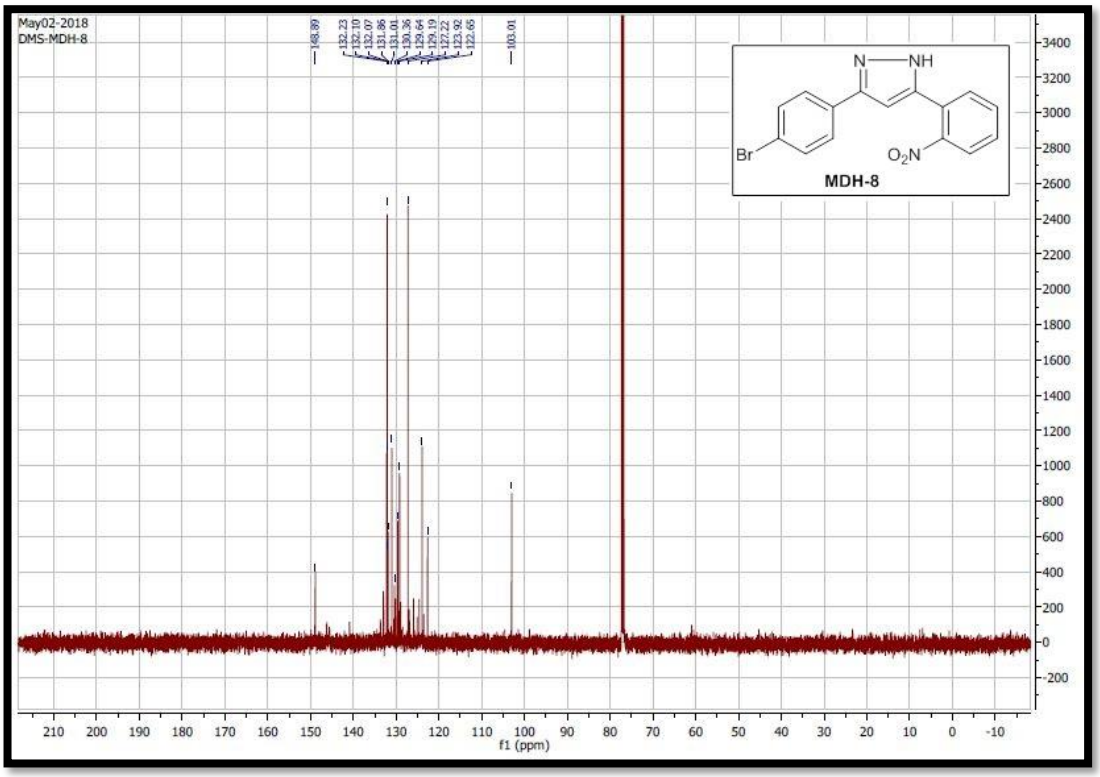
UV spectra of MPH-1 and MDH-1



UV spectra of MPH-8 and MDH-8



^{1}H NMR spectra of MDH-8

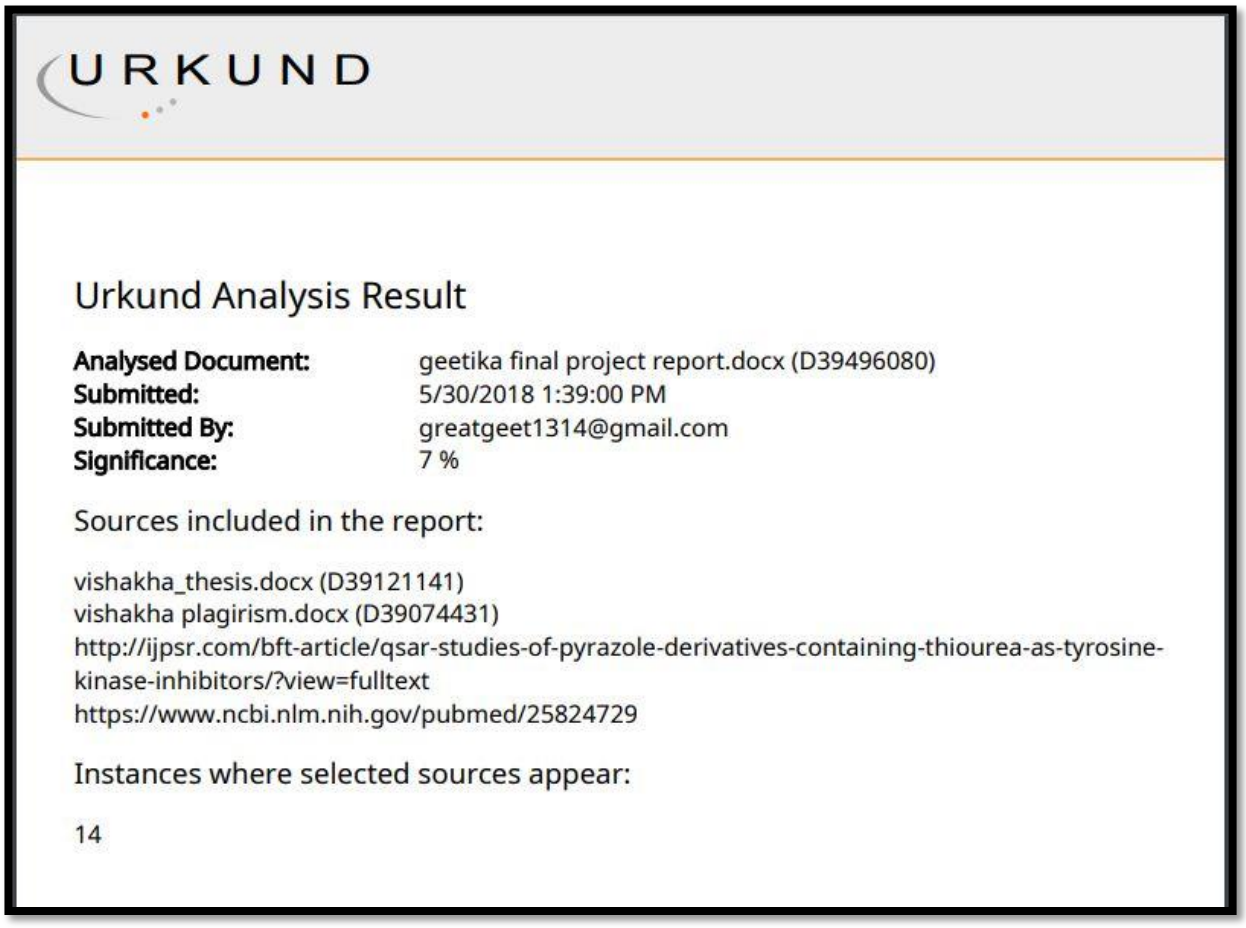


^{13}C NMR spectra of MDH-8

APPENDIX-B

Plagiarism Report

Plagiarism report



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Urkund Analysis Result

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Similarities were found in the reviewed literature and proper references have been given.