SYNTHESIS AND ANTIPROLIFERATIVE ACTIVITY OF PYRAZOLE-BASED HETEROCYCLES

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By

Vishakha Pandey
(Reg.no.16mpharm06)

Supervisor
Dr. Raj Kumar

Department of Pharmaceutical Sciences and Natural Products

School of Basic and Applied Sciences

Central University of Punjab, Bathinda

June 2018

DECLARATION

I declare that the thesis entitled **Synthesis and Antiproliferative Activity of Pyrazole-Based heterocycles** has been prepared by me under the guidance of Dr. Raj Kumar, Associate Professor, Department of Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences. No part of this thesis has formed the basis for the award of any degree of fellowship previously.

Vishakha Pandey

Department of Pharmaceutical Sciences and Natural Products

School of Basic and Applied Sciences,

Central University of Punjab,

Bathinda, India -151001

Date:

CERTIFICATE

I certify that Vishakha Pandey has prepared her thesis entitled **Synthesis and Antiproliferative Activity of Pyrazole- Based heterocycles** for the award of M.Pharm. Degree in Medicinal Chemistry under my guidance. She has carried out this work at Department of Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab.

Dr. Raj Kumar

(Supervisor)

Associate Professor & Head of Department

Department of Pharmaceutical Sciences & Natural Products,

Central University of Punjab,

Bathinda-151001

Date:

ABSTRACT

Synthesis and Antiproliferative Activity of Pyrazole- Based heterocycles.

Name of student : Vishakha Pandey

Registration Number : 16mpharm06

Degree for which submitted : M.Pharm. (Medicinal Chemistry)

Name of supervisor : Dr. Raj Kumar

Department : Pharmaceutical Sciences and Natural Products

School of Studies : School of Basic and Applied Sciences

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ABSTRACT

Among the various heterocyclic compounds pyrazole and its derivatives have occupied wide range of biological and pharmacological activities. These were observed for their modes of function in the inhibition of topoisomerase and DNA repair. DNA topoisomerases usually modify DNA topology by their ability to break and reseals both its strands. Which were leads to DNA replication, transcription processes. It helps as a vital targets for numerous chemotherapeutic agents. The potency of topoisomerase inhibitors looks to be diminishing due to drug resistance and lack of efficacy. Thus, after long glimpsing the current scenario was made in order to develop topoisomerase inhibitors with completely new scaffold or alteration or modification in the existing scaffold. We herein report design and synthesis of pyrazole based compounds as topoisomerase inhibitors. The synthetics were evaluated for their in vitro anticancer activity against MDA-MB 231 breast cancer cell line.

Vishakha Pandey Dr. Raj Kumar

Dedicated to my Loving Parents

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LIST OF ABBREVATION

S.No.	Full Form	Abbreviation
1.	Human topoisomerase	Htopo
2.	Nuclear magnetic resonance	NMR
3.	Adenosine triphosphate	ATP
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 bank	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

CHAPTER-1
INTRODUCTION

1. **Introduction**

Cancer is a disease caused by an uncontrolled, rapid and pathological proliferation of abnormal cells in the body(Cairns & Mak, 2016). It involved more than hundred diseases having common features of angiogenesis, excessive proliferation, and metastatic properties. Cancer is the second leading cause of death in the nation after the cardiovascular disease, it causes 550,000 deaths a year(Tsai et al., 2015). There are 200 different types of cancer were classified according to the type of cell which were initially affected(Sharma, Kumar, & Bhushan, 2014). In India, the number of cancer was increasing. According to WHO cancer report, most common is the breast cancer which is more than 255,000 cases were expected in India in 2017 and after that lung cancer and the prostate cancer are the most common cancer. In Punjab, lung cancer is the most common cancer after that breast cancer is the second and third is cervical cancer(Chakrabarty, Pai, Ranjith, & Fernandes, 2017). The treatment of cancer cure by surgery after that radiation therapy, chemotherapy, and other related therapies are immunotherapy, targeted therapy or hormone therapy. Besides every treatment, even chemotherapy is associated with side effects like resistance, solubility issue and low efficacy(Niazi, Rabizadeh, & Bredesen, 2018). In the last few years, various heterocyclic derivatives produce anticancer impact in various types of cancer through inhibiting cell growth and induction of cell differentiation and apoptosis(Karrouchi et al., 2018). Among the various heterocyclic derivatives, pyrazole derivatives are related with potent anticancer feature due to their inhibition of topoisomerase (topo) I/II, EGFR, VEGF, telomerase, etc.(Alam, Alam, & Panda, 2018).Topoisomerase is an enzyme which alters the supercoiled form of a DNA molecule. It is the second most abundant protein after HDAC. It is of two type htopo I and htopo II (gyrase). In htopo I, it cuts phosphatester bond on one DNA strand, rotate the broken DNA freely around the other strand to relax the constraint and releases the cut. In htopo II, it cuts phosphatester bond on both strands of DNA, releases the supercoil constraint and reform the phosphatester bonds.

In this research work, we have synthesized pyrazole based heterocycles molecules as dual topo I and topo II inhibitors and screened them against MDA-MB- 231 breast cell line to assess their antiproliferative activity.

CHAPTER-2
LITERATURE REVIEW

2. Literature Review

2.1 Cancer and Mortality

Cancer is leading cause of global mortality and the second most common cause of death(Pan et al., 2017). Cancer can start anywhere in the body, which is made up of trillions of the cell. When cancerous cell divided it leads to the formation of tumor in the form of lumps. Tumor can be of two type: i) benign tumor, which does not spread into nearby tissue and ii) malignant, the spread of cells to the distant sites, usually via blood stream, lymphatic system, or through body spaces known as metastasis(Ruggieri, Angelini, Vanel, & Picci, 2017). There are various internal and external factor which were responsible for cancer such internal factors are extreme hormonal changes, inherited genetic disorders, immunity, and family history and external factors are the infectious organism, chewing tobacco, exposure to harmful radiations as well as chemicals (O'Leary, Suri, & Gross, 2018). Cancer having High mortality rates in worldwide(Hashim et al., 2016). It has been reported that the over 60% of death happened due to low medium resource economics due to poverty, environmental pollution, poor medical and health systems(Siegel, Miller, & Jemal, 2016). The world health Organization reported that worldwide total morbidity and mortality was 6.2 million in 1997, 7.4 million in 2004, and 7.6 million in 2008, it means 13% of all deaths were due to cancer and that the global cancer rate could increase by 50% to 15 million new cases by 2030(Valery et al., 2018). Recent data suggested that a total of 1,660,290 new cancer cases and 580,350 cancer deaths in the United States in 2015. According to latest report of world cancer, from the world health organization (WHO), women are more in number to develop cancer annually. In India out of 1.2 billion population 1 million new cancer cases detected each year (Ferlay et al., 2017). Presently the growth of cancer is increasing day by day and now it become the third top root of deaths in developed nations(Ashraf & Jamil, 2016). On the world cancer day, WHO published the current cancer statistics, in 4th February 2016 death of 8.2 million of people with cancer annually(Heron & Anderson, 2016). In the recent scenario, Punjab state highlights that the Malwa region, recognized as the cancer belt, has the maximum average of 136 cancer patients per 1 lakh people which is more than the national average of 80 per lakh(Nanda, Kumar, Kumar, Behal, & Nanda, 2016). In Punjab region, lung cancer, breast cancer and cervical cancer claim more number of death. Four

districts that increase the cancer incidence list are from Muktsar, Mansa, Bathinda and Ferozepur districts in which Muktsar district fares the worst and it is followed by Mansa, Bathinda and Ferozepur districts. In the Majha region at Tarn Taran district has the minimum number of cancer patients- 41 per 100,000 people. The rich Doaba region, including, Jalandhar, Hoshiarpur, Phagwara, Kapurthala, has confirmed 88.1 cancer patients per lakh of the population(Godara, 2015). A hospital report figured out that the highest number of cancer patients from Bathinda region of Punjab (Kaur, 2015).

2.2 Hallmarks of cancer

Cancer is not a single disease but group of diseases, which involve abnormal cell growth with the potential to spread. The National Cancer Institute defines cancer as “ a term of disease in which cell divides without control” (Selhi, Narang, Singh, Sood, & Kaur, 2017). The hallmarks of cancer comprise of ten biological capabilities developed during the development of human tumor as shown in figure 2.2. Each capability having a distinct functional role in supporting the development, progression, and persistence of tumors. It includes evading growth suppressors, avoiding immune destruction, enabling replicative immortality, sustaining proliferative signaling etc.(Hanahan, Weinberg, & Hanahan, 2017).

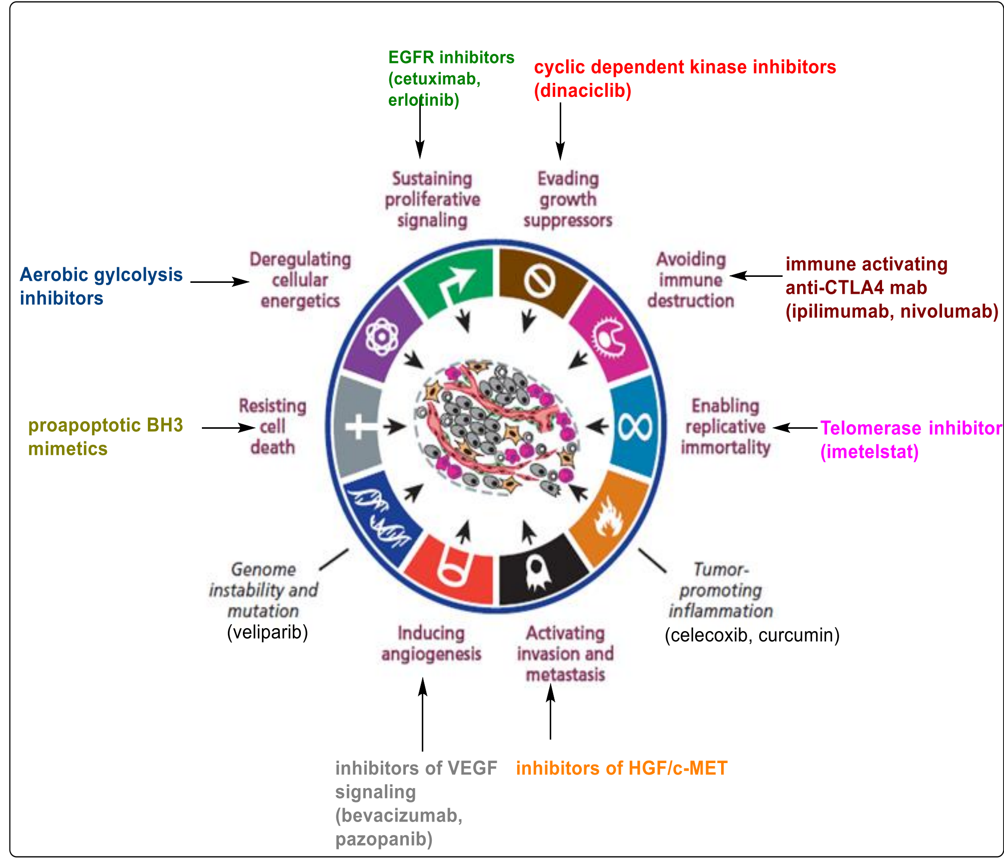


Figure 2.2: The biological hallmarks of cancer (Hanahan et al., 2017)

2.3 Chemotherapy in cancer treatment

The main treatment of cancer is chemotherapy, which means treatments with drugs. It uses the special drug to kill cancer cell in the body. These drugs are basically cytotoxic, hormonal therapies, biological therapies and target therapies for cancer(Zhou et al., 2017). Most cancers are treated with surgery, chemotherapy or radiotherapy. Other treatments, such as hormone therapy and immunotherapy, can also be used for some types of cancer. Sometimes targeted therapy is used instead of chemotherapy. Chemotherapy can be used to destroy cancer cells, stop cancer cell from spreading, slow the growth of cancer cells. There are numerous factor which were influence tumor response to chemotherapy are tumor size, cell resistance to drugs, amount of chemotherapy given, and condition of a patient. Chemotherapy mainly used in four clinical setting 1) Primary induction treatments for advanced diseases or for cancer for which there are no other effective treatments.2) neoadjuvant treatments for patients who present with localized disease, for whom local form of therapy such as, surgery and radiation. 3) Adjuvant treatments to local treatments modalities,

including surgery and radiation therapy. 4) Direct instillation into sanctuary sites or by site-directed perfusion of a specific region of the body directly affected by cancer(Chu & DeVita Jr, 2017). Chemotherapy mainly given in combination therapies to decrease the adverse effect and resistance and increase the effectiveness of the drug(Thiboldeaux & Golant, 2012). The growth of cancer can be cure by using chemotherapy but it kills cancer as well as normal cells too and these affected normal cell gives unpleasant adverse effects. Based upon the different type of therapy it have different side effect (Moussa, Watson, & Halushka, 2013).In the particular treatment, different people react in many other ways. A small number of people have very few side effects or no side effects. The acute toxicities have major sites for example gastrointestinal tract, hair follicles and proliferating tissues such as bone marrow, etc.(Davies & Reid, 2014).

Depending upon the cancer type, there are a different route of administration for chemotherapy drugs, for example oral chemotherapy, intramuscular chemotherapy, subcutaneous injection, intravenous chemotherapy, and intrathecal chemotherapy. The classical chemotherapy which is based on non-targeted approach having drugs which are cytotoxic, suppresses hematopoiesis, and induce renal, vascular and hepatic injury e.g. alkylating agents (cisplatin, chlorambucil, etc.), topoisomerase I (topotecan, irinotecan,etc.) Topoisomerase II (mitoxantrone, etoposie, etc.) antibiotics (doxorubicin, daunarubicin, etc.). Antimetabolites (methotrexate, mercaptopurine, 5 fluorouracil, etc.) and (Harvey & Khuri, 2017).as shown in table 2.3.1

Table 2.3.1: various cytotoxic drugs

Class	Example	Cancers used against
Alkylating agents	chlorambucil; cisplatin	Breast Lymphoma
Topoisomerase inhibitor 1	topotecan; Irinotecan	Lymphomas, leukemias, refractorysolid tumors.
Antimetabolites	Methotrexate; 5-fluorouracil	Colorectal Leukemias Breast
Topoisomerase inhibitor 2	Mitoxantrone	acute leukemia, lymphoma, prostate cancer

Among the various cytotoxic drugs, these drugs reach almost all cells in the body and they kill cancer cells as well as healthy cells too. This is why chemotherapy has an adverse effect. Anticancer drugs have many problems like efficacy,

resistance, specificity and numerous side effect for example alkylating agents having pulmonary toxicity, neurotoxicity, hemorrhagic etc. antibiotics have (UTI) urinary tract infection, kidney function. Topoisomerase shows alopecia, bone marrow suppression, heart damage due to high dose etc. and some antimetabolites having gastroenterititis, stomatitis, anaphylaxix,etc. (Hausheer, 2016).Side effect associated with chemotherapeutic drugs as shown in figure in 2.3

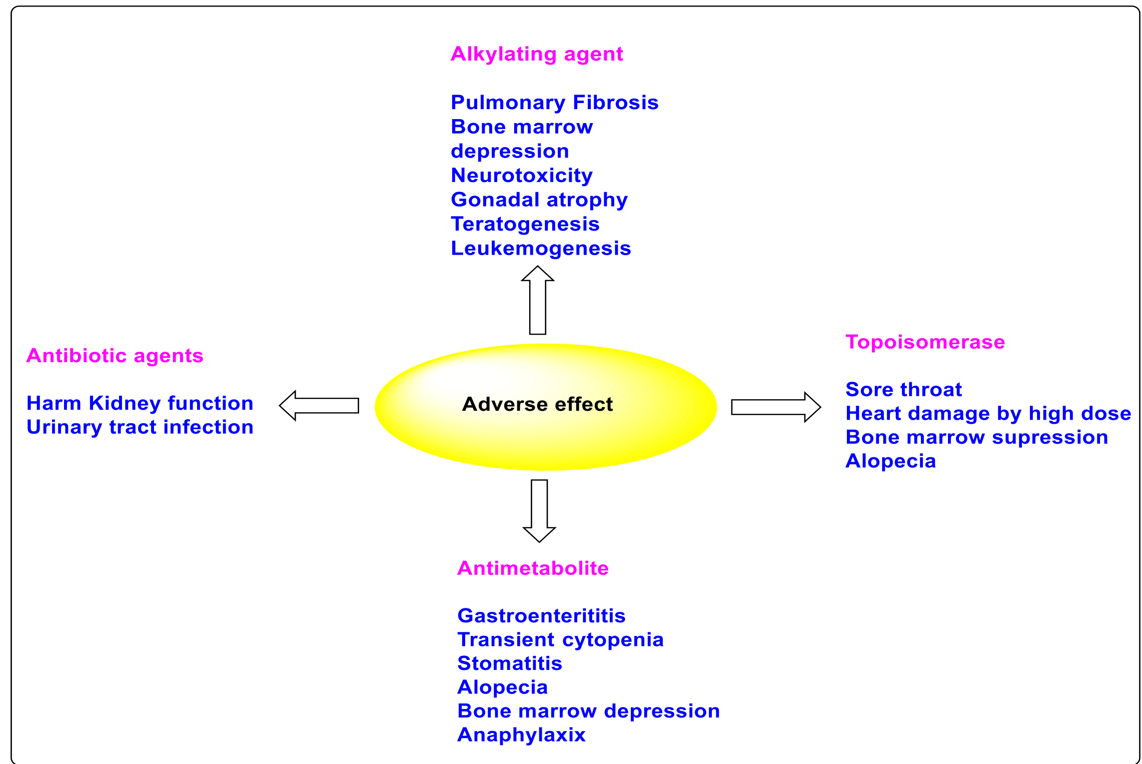


Figure 2.3 Adverse effect of anticancer drugs

Cancer has the ability to become resistant for various types of drugs, there are an different sort of reasons that lead to failures in cancer chemotherapy(Suresh, Ali, Ahmad, Philip, & Sarkar, 2016). A few activities that allow tumor cells to survive the chemotherapy are: (a) enzyme inactivation,(b) increased efflux of drug,(c) increased tolerance to DNA damage,(d) high anti-apoptotic potential, and (e) decreased permeability(Rueff & Rodrigues, 2016). Further than various side effect ,(MDR) multi-drug resistance is the major problem with anticancer chemotherapy and it is associated with over expression of (ABC) ATP-binding cassette transport ,more expression anti-apoptotic and lesser expression of pro-apoptotic genes, increase expression of specific tubulin isotypes and decrease expression of topoisomerase,etc.(Settleman, 2016).

Targeted Anticancer Therapy

Targeted drug therapies are vastly improved endured than traditional therapies. These targeted drug therapies have been a significant innovative in rational drug design and a good way in improving cancer treatment (Pérez-Herrero & Fernández-Medarde, 2015). In 1997 the first molecular targeted cancer drug which is FDA approved, rituximab (PDB)-4KAQ is an type of antibody therapy, dramatically transformed the oncology drug market(Rossig, Juergens, & Berdel, 2011).

Targeted therapies aim to attack these pathways

(a) Preventing the productions of hormone involved in the tumor growth.

(b) Blocking the growth of blood vessels that provide nutrients for cancer cell growth.

(c) Promoting apoptosis

(d) signaling the immune system to kill cancer cell(Borisy et al., 2016).

Targeted chemotherapy includes various anticancer agents acting on multiple cellular targets which involves in cancer progression such as receptor and non-receptor kinases (EGFR and VEGFR family),nuclear proteins /enzymes (HDAC, tubulin, topoisomerase I and II) as shown in the list table 2.3.2 (Raymond, Faivre, & Armand, 2000)

Table 2.3.2: Targeted Anticancer Therapy

Targets	Drugs	Therapeutic Indications
HDAC	Vorinostat, Romidepsin,	CutaneousT-cell lymphoma
Non receptor tyrosine kinases (EGFR and VEGFR family),	Imatinib Dasatinib Erlotinib Gefitinib	Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia chronic myeloid leukemia (CML) Breast cancer, colon cancer, pancreatic cancer
Serine/ threonine kinases	Trametinib Vemurafenib	Metastatic melanoma progressive tumors of the digestive system
Receptor tyrosine kinases	Sunitinib Lenvatinib Olaratumab	Advanced renal cell carcinoma Soft tissue sarcoma
Microtubules	Paclitaxel	Breast cancer, ovarian cancer, and lung cancer
Topoisomerase I Topoisomerase II	Irinotecan, Topotecan Etoposide	Cancers of the colon and rectum Small cell lung cancer

Proteasome	Bortezomib, Carfilzomib, Ixazomib	Multiple myeloma and mantle cell lymphoma Multiple myeloma

2.4 Topoisomerase as Drug targets

Topoisomerases is an nuclear enzyme that control topological problems in genomic DNA, which can result from DNA replication, transcription and repair(Cuya, Bjornsti, & van Waardenburg, 2017). The enzyme classified into human topoisomerase I (htopo I) and human topoisomerase II (htopo II). The DNA topoisomerase I break the single strand of DNA and topoisomerase II relax the super coiled of DNA and break the double strand of DNA having phosphate background and needs ATP(S. H. Chen, Chan, & Hsieh, 2013). In htopo II structure, the catalytic process involved the opening and closing of molecular gates which are regulated in ATP binding. The ligand binding to the ATPase domain of htopo II leads to the inhibition of ATP-regulated opening and closing of the molecular gates and hence suppresses the activity of htopo II(Joshi et al., 2016). The htopo II classified into two classes, such as htopo IIα and htopo IIβ, both are isozymes having similar catalytic site and different biological functions. The htopo IIα involved in cell division and htopo IIβ associated in cell differentiation(Ganapathi & Ganapathi, 2013). The overexpression of topoisomerase enzymes leads to cancer(Giles & Sharma, 2005). A huge number of Topo targeted drugs are currently identified that interact directly with the DNA or inhibit the topological relaxation of the DNA(Palchaudhuri & Hergenrother, 2007). The topoisomerase inhibitor interact with the topoisomerase enzymes and show various responses that contain inhibitors selectively affecting the relegation of DNA, thereby changing Topo target enzyme into a DNA damaging agent(Hande, 1998). The Topo inhibitors might be attributed to inhibition of the ligation step during the cell cycle progression thereby generating single and double stranded breaks that finally harm the integrity of the genome thereby inducing apoptosis. Therefore the topo inhibitors are designed specifically to interfere the action of topo enzymes that are involved in the breaking and rejoining the phosphodiester backbone of DNA strands throughout the regular cell cycle. There are various topo inhibitors that contain topo poisons for example:

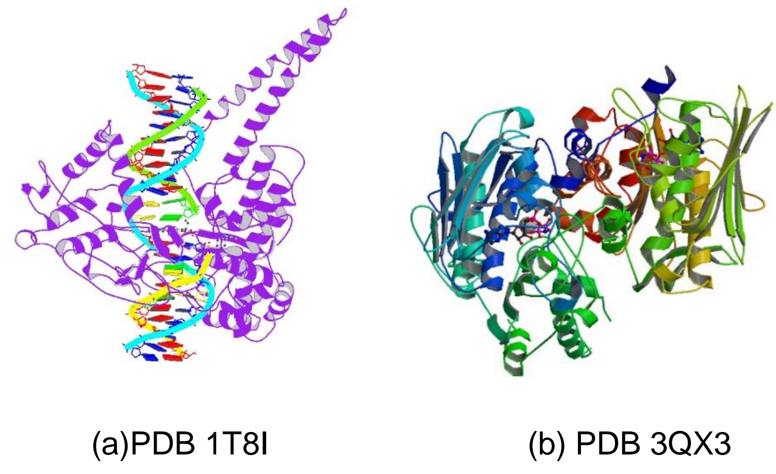
topo I inhibitor (Amscarine, Etoposide, fluoroquinolones, camptothecin) and topo II inhibitor (etopoide), these drugs are contemporary used in the treatment of cancer(Dwarakanath, Khaitan, & Mathur, 2004). Among the various drugs which are available as topo inhibitors, suffer from resistance issue and toxicity during monotherapy. In topo I inhibitor, myelosuppression is the primary dose-dependent toxicity(Pommier et al., 1994) and in topo II inhibitor, myelosuppression and gastrointestinal toxicity releated to short term toxicity and cardiac toxicity and secondary leukemia releated to long term toxicity(Seiter, 2005).

2.4.1 Classification of topoisomerases

Topoisomerase enzymes classified into two types: topoisomerase I (topo I) which cut single strand of DNA and further classified into type I subfamily member (if protein link to 50 phosphate) and type II subfamily member (if protein link to 30 phosphate) and topoisomerase II which cut double strand of DNA(Champoux, 2001).

Type I topoisomerase topo I was discovered by James Wang in 1971. It is nicking- closing enzymes are monomeric 100kd proteins that are widespread in both prokaryotes and eukaryotes(deJonge, 1999). In bacteria topo I can remove negative supercoils. The topo I does not required energy from ATP hydrolysis. It produce break in single strand DNA. The crystal structure of topo I (PDB 1T8I) as shown in figure 2.4 (a)

Type II topoisomerase topo II was discovered by Martin Gellert in 1976 and isolated from E.coli and named as DNA gyrase are 375kd proteins that consist of two paired of subunit(Cozzarelli, 1980). This enzyme required energy from ATP hydrolysis. In topo II it produce break in double strand DNA(Smith, Cowell, & Austin, 2018). Topo II further classified into Topo IIα and Topo IIβ. The crystal structure of topo II (PDB 3QX3) as shown in figure 2.4 (b) (Wu et al., 2011).



(a)PDB 1T8I (b) PDB 3QX3

Figure 2.4 : The crystal structutre of topo I (a) and topo II (b) (http://www.rcsb.org)

In topo I (PDB-1K4T) the important amino acid that are present in binding site Arg503 and Asp479 whereas in topo II (PDB: 3QX3) Lys532 and Asp533.Further, most of the catalytic topoII inhibitors occupy the highly conserved Walker A motif consisting Arg162, Asn163, Gly164, Tyr165, Gly166 and Ala167 of the ATPase domain. It has been understood that rapidly proliferation tumor cells express the enzyme Topo I/Topo II at 25-300 times higher levels than those of the quiescent cells recommending them as biological targets as proteins for new anticancer agents (Joshi et al., 2016).

The mode of action of topo I and topo II as shown in figure 2.5(a) topo I makes a nick in one strand of DNA molecule, and passes the intact strand through the nick and release the gap(Pommier, Pourquier, Fan, & Strumberg, 1998). (b) Topo II makes a double strand break in the double helix, creating the gate through which a second segment of the helix is passed(Burden & Osheroff, 1998).

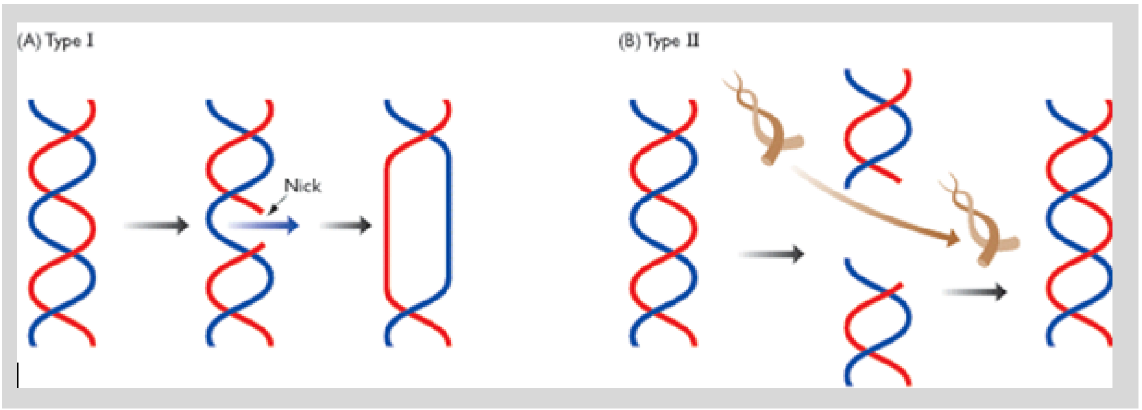


Figure 2.5 Mode of action of topo I and topo II

2.4.2 Topoisomerase inhibitors

Topoisomerase inhibitor are the chemotherapy medication which were used in the treatment of various cancer.These inhibitor interfere with enzyme called topoisomerase and lead to cell death(Sinha, 1995). The DNA topoisomerase I and II are essential enzymes for transcription, replication and mitosis. The following drugs that are able to inhibit these enzymes are shown in figure: 2.6 Topoisomerase I (topo I) inhibitors: irinotecan, topotecan, camptothecin and Topoisomerase II (topo II) inhibitors: etoposied, amsacrine,(these are semisynthetic derivatives of epipodophyllotoxins, alkaloids naturally occurring in the root of American Mayapple (Podophyllum peltatum), doxorubicin, daunorubicin, mitoxantrone are called topoII poisons as they use potent cytotoxic effect by stabilizing the DNA –enzyme complex.(K Kathiravan, N Kale, & Nilewar, 2016).

Camptothecin

Irinotecan

Topotecan

Topoisomerase I inhibitors

Doxorubcin

Mitoxantrone

Etoposide

Topoisomerase II inhibitors

Figure 2.6: Topoisomerase inhibitors as anticancer compounds

Different classes of DNA topoisomerase inhibitors are shown in the table 2.4.2

S.NO.	Inhibitor	Target enzyme	Therapeutic value
1.	Coumarin (e.g.- novobiocin)	Topoisomerases IV and DNA gyrase	Antibiotics
2.	Camptothecin(e.g.- topotecan)	Human topoisomerase I	Anticancer drug
3.	Amsacrine	Human topoisomerase II	Anticancer drug
4.	Epipodophyllotoxins (e.g.- teniposide)	Human topoisomerase II	Anticancer drug
5.	Quinolones(e.g.- ciprofloxacin)	Topoisomerases IV and DNA gyrase	Antibacterial agents

2.4 Heterocycles in development of anticancer drugs

In the recent year, large number of heterocyclic compounds having both synthetic and natural products are pharmacological active and are in clinical use(Shukla, Verma, & Mishra, 2017). In the branch of organic chemistry, heterocycles are an important and unique class of compounds and have wide range of physical, chemical and biological properties spanning a broad spectrum of reactivity and stability(Abdallah et al., 2017). The heterocyclic compounds are widely distributed in nature and play an important role in metabolism because of their structural subunit exist in many natural products for examples, antibiotics, vitamins, hormones, alkaloids, agrochemicals, dyes etc.(Arora, Arora, Lamba, & Wadhwa, 2012). There are various heterocyclic compounds, the nitrogen

containing compounds produce anticancer effect in various type of cancer through inhibiting of cell growth and induction of cell differentiation and apoptosis(Bisi et al., 2017). Among the various heterocyclic compounds, pyrazole derivatives are classified at higher position. In 1959, the first natural pyrazole, 1-pyrazolyl-alanine as shown in figure 2.7, was isolated from seeds of watermelon Citrullus lanatus after 75 years(Akhtar, Khan, Ali, Haider, & Yar, 2017)

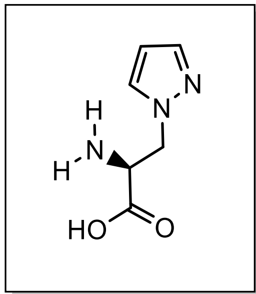


Figure 2.7. 1-pyrazolyl-alanine

Pyrazole is an important member of heterocyclic compound having two adjacent nitrogen in five member ring framework in which one is basic and other one is neutral in nature. The partial reduce form of pyrazole is called as pyrazoline while complete reduce form is called pyrazolidine(Küçükgüzel & Şenkardeş, 2015). The term pyrazole was given by "Ludwig Knorr". Pyrazole shows various activities such as anti-inflammatory, analgesic, antiviral, anti-convulsant, anti-allergic, anti-tubercular, anti-fungal ACE inhibitors, etc.(Faria et al., 2017). Among the various biological activity pyrazole have potent anticancer activity and various targets receptors such as protein kinase inhibitor, topoisomerase I/II, tyrosine kinase, Aurora-A kinase, tumor growth factor (TGF), cyclin dependent kinase (CDK) and fibroblast growth factor (FGF), which are important for the management of cancer(Kumar, Saini, Jain, & Jain, 2013). There are some chemical structure of pyrazole-based (markted faces) anticancer drugs(Fahmy et al., 2016) as shown in figure 2.8

Figure 2.8: Pyrazole based anticancer drugs

Among the various anticancer drugs, pyrazole based topoisomerase II inhibitor as anticancer are teloxantrone HCl and losoxantrone, both are in clinical trials. The teloxantrone HCl is an anthrapyrazole antineoplastic antibiotic. Teloxantrone introduces into DNA and interects with topoisomerase II, thereby inhibiting DNA replication and repair as well as RNA and protein synthesis(T.-C. Chen et al., 2015). The second is losoxantrone is an anthrapyrazole –based antineoplastic antibiotic. Losoxantrone intercalates into DNA, induce single and double-stranded DNA breaks and inhibits topoisomerase II, thereby inhibiting DNA replication and repair as well as RNA and protein synthesis. Losoxantrone is less cardiotoxic than doxorubicin (Begleiter et al., 2006). As shown in figure 2.9

topoisomerase 2

Teloxantorne HCl

losoxantrone

Figure 2.9: Topoisomerase inhibitors having pyrazole ring

CHAPTER-3
RATIONALE

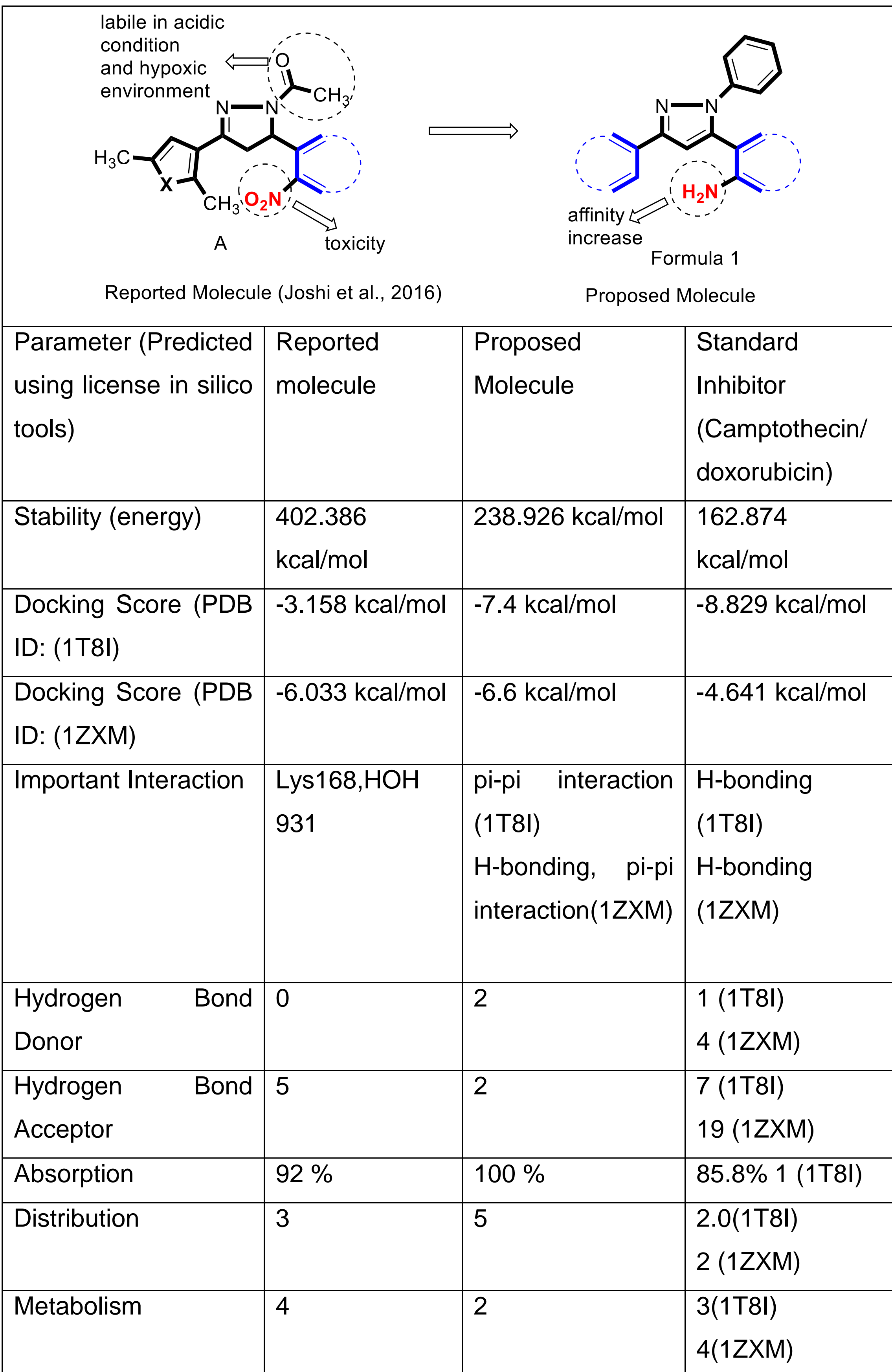
3 Rationale

We thought of designing new compounds as topo inhibitors after looking into the drawbacks of N-acetyl pyrazolines as topo inhibitors (Joshi et al., 2016) that include. (i) The N-acetyl group may act as toxicophore due to its electrophilic reactive nature for e.g.paracetamol cause hepatotoxicity. (Anastas & Maertens, 2017). (ii) The N-acetyl group may get cleaved under the acidic and hypoxic environment of solid tumors. (iii)The -NO_2 group is also known to be a potent toxicophore (for e.g. Chloramphenicol, precipitates gray baby syndrome).

Considering the above drawbacks, Formula 1 can be designed which includes: (i) replacement of acetyl group with phenyl group because acetyl group is labile in acidic condition and hypoxic environment (ii) Incorporating free amino group instead of -NO_2 group in the reported entity to increase the affinity of the compound and toxicity of existing molecule; (iii) converting pyrazoline to its stable form pyrazole.

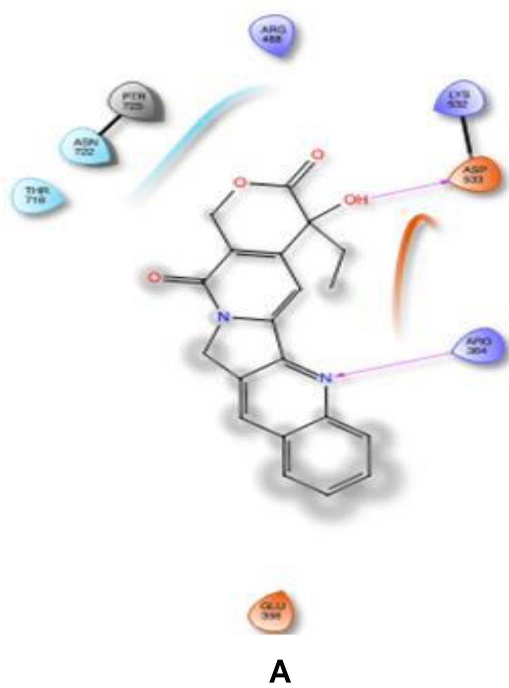
To further strengthen our rationality, we performed energy related stability studies using MM9 tool (ChemBio 3D Ultra14.0), molecular docking (Maestro 11.1) and ADME prediction on proposed and reported molecules. The brief results of prediction are compiled in table 3.

Table 3: Results of following Parameters

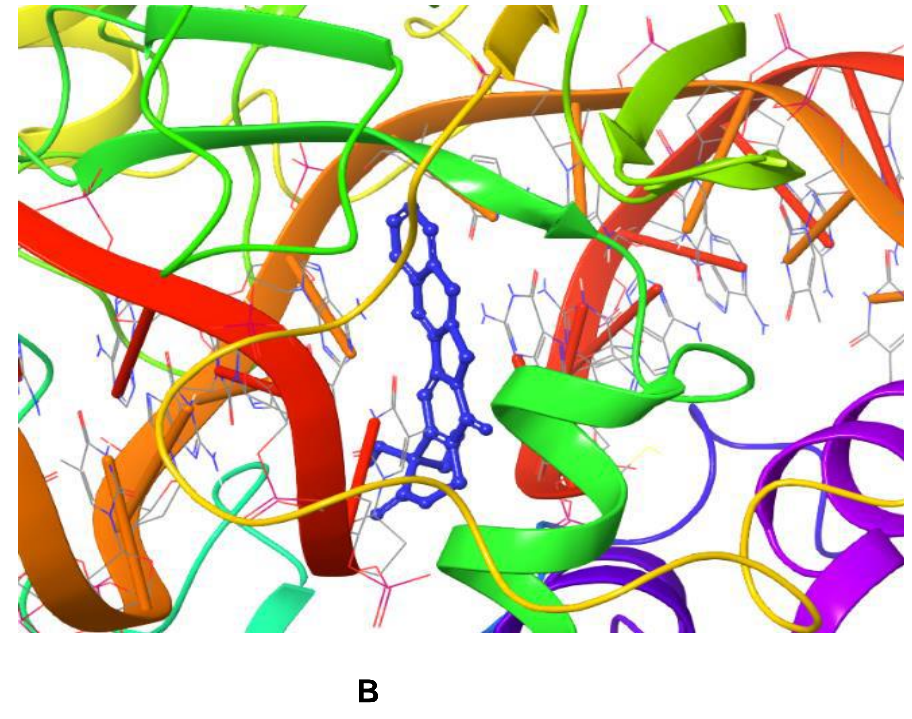


Parameter (Predicted using license in silico tools)	Reported molecule	Proposed Molecule	Standard Inhibitor (Camptothecin/ doxorubicin)
Stability (energy)	402.386 kcal/mol	238.926 kcal/mol	162.874 kcal/mol
Docking Score (PDB ID: (1T8I)	-3.158 kcal/mol	-7.4 kcal/mol	-8.829 kcal/mol
Docking Score (PDB ID: (1ZXM)	-6.033 kcal/mol	-6.6 kcal/mol	-4.641 kcal/mol
Important Interaction	Lys168,HOH 931	pi-pi interaction (1T8I) H-bonding, pi-pi interaction(1ZXM)	H-bonding (1T8I) H-bonding (1ZXM)
Hydrogen Bond Donor	0	2	1 (1T8I) 4 (1ZXM)
Hydrogen Bond Acceptor	5	2	7 (1T8I) 19 (1ZXM)
Absorption	92 %	100 %	85.8% 1 (1T8I)
Distribution	3	5	2.0(1T8I) 2 (1ZXM)
Metabolism	4	2	3(1T8I) 4(1ZXM)

The results from table 3, portrayed that the energy of proposed molecule is lesser than the reported molecule showed the better stability as compared with the standard molecule. From the docking studies the proposed molecules show better dock score in topo I (-7.4 kcal/mol) and in topoII (-6.6 kcal/mol) as from the reported molecule as compared with standard which indicates that the synthesized molecule have greater affinity as shown in figure 3.1 and 3.2, repectively. The proposed molecule showed 100% human oral absorption as from the reported molecule compared with standard which indicate that the compound have drug able property. However, the distribution show greater and having less metabolism reactions as reported molecule, compared with the standard which prolong the molecule action. These above studies indicate that the proposed molecule have drug able property and also may be used as topo inhibitor.

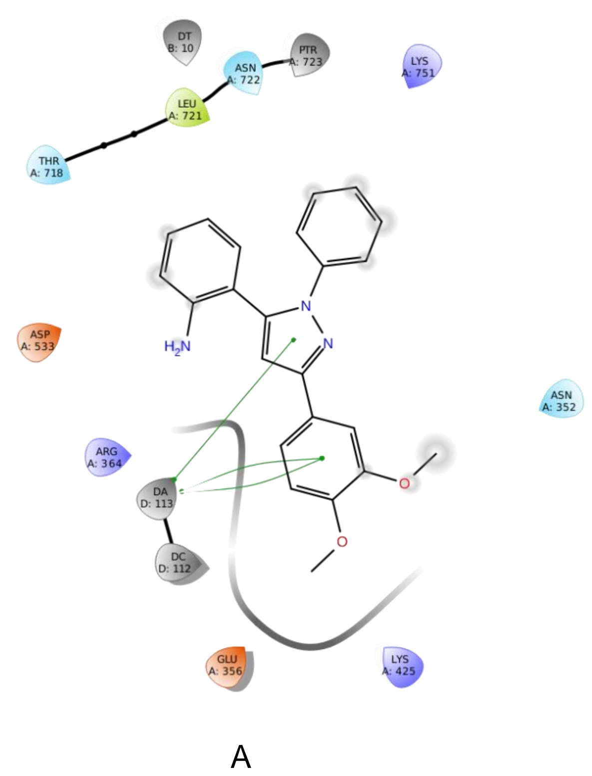


A

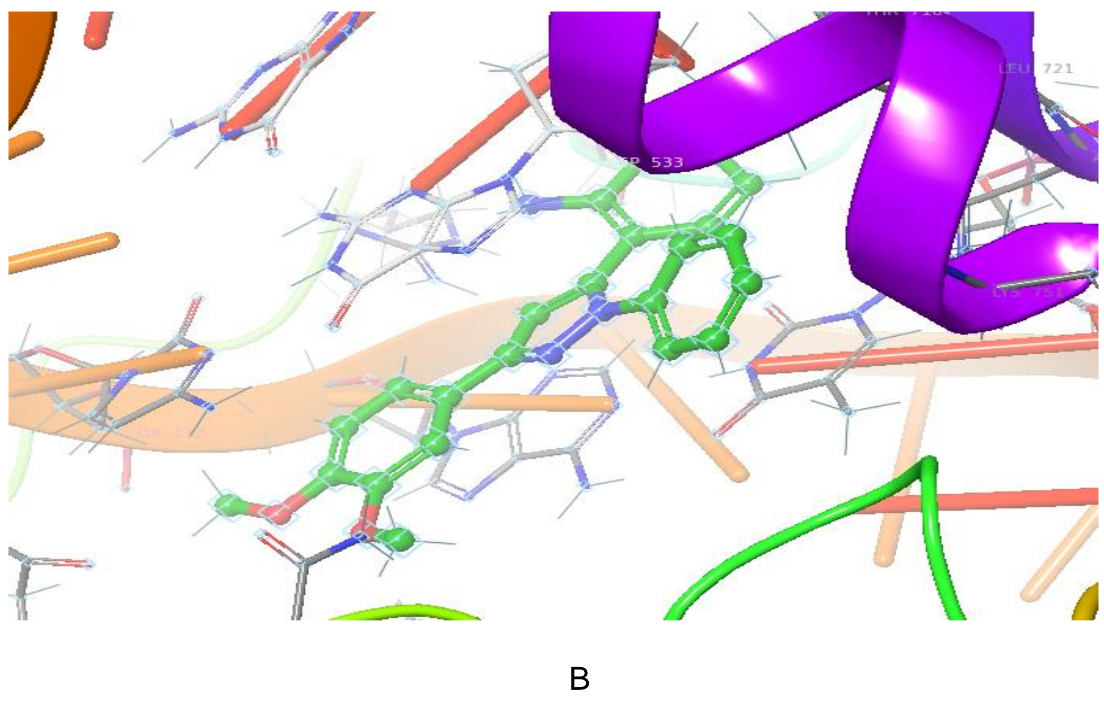


B

Figure 3.1(a): Interaction of Camptothecin with htopoI **(1T8I)**



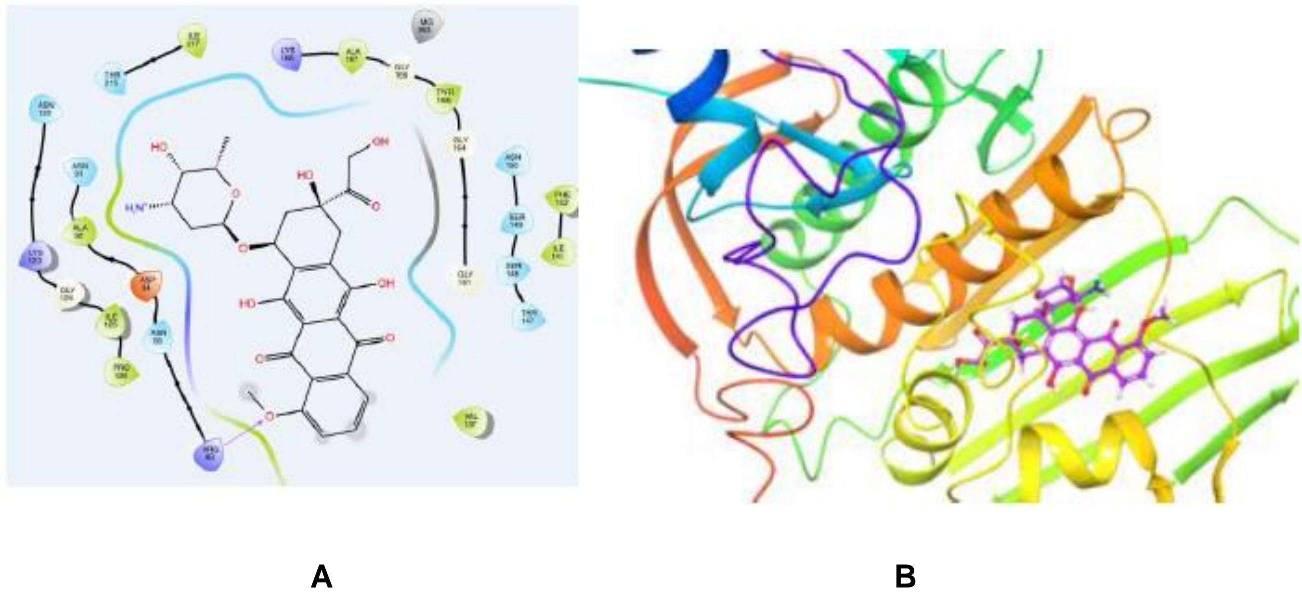
A



B

Figure 3.1 (b): Interaction of 4b with htopo I **(1T8I)**

S.No	CODE	DOCK SCORE
1.	CAMPTOTHECIN	-8.829
2.	4b	-7.4



A **B**

Figure 3.2(a): Interaction of doxorubicin with htopoII **(1ZXM)**

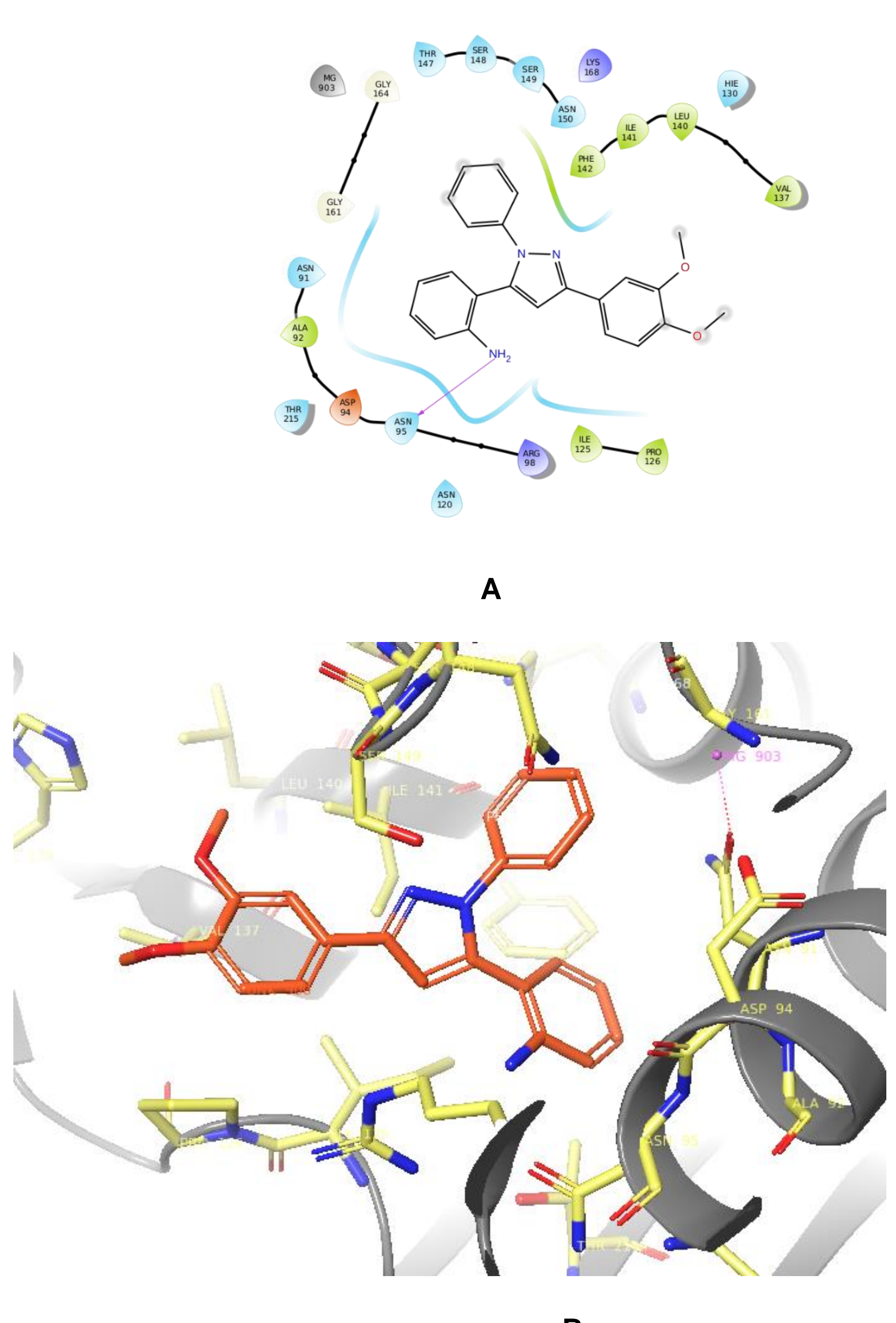


Figure 3.2 (b): Interaction of 4b with htopo II **(1ZXM)**

S.No	CODE	DOCK SCORE
1.	DOXORUBICIN	-4.641
2.	4b	-6.6

CHAPTER-4
OBJECTIVES

4. Objectives

Based on the Rationality we have set following objectives

A. To synthesis pyrazole based heterocycles as antiproliferative agents.

B. To assess in vitro antiproliferative activity of synthesized compounds using MTT assay.

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 Pt. Ltd. Avra Synthesis (AR/GR quality) were used without any additional purification.
2. For weighing purposes, Sartorius Analytical balance (BSA224S-CW) was used. ILMVAC Rotary evaporator was used for evaporating solvents and JSGW Heating mantle for reflux reactions.
3. The progress of reaction was monitored by TLC, petroleum ether/ethyl acetate or chloroform/methanol used as mobile phase on pre-coated TLC plates (Merck) JSGW UV/fluorescent cabinet or iodine chamber.
4. Melting points were recorded on Stuart melting point apparatus (SMP-30) with open glass capillary tubes and were uncorrected.
5. The compounds were further purified using column chromatography silica gel size (60-120)
6. The Mass (EI) spectra of compounds were recorded on Shimazdu GCMS-QP2010 at Central instrumental laboratory (CIL), Central University of Punjab.
7. 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.
8. UV spectra of compounds were recorded at Department of Pharmaceutical Sciences and Natural Products, 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, Hisar (Avanu III) in $CDCl_3$/ d_6-DMSO on a Bruker Advance II 400 MHz and 100 MHz respectively using TMS (δ = 0) as an internal standard.

5.1.1 General procedure for the synthesis of chalcones (1a-1e)

To a mixture of substituted aldehyde (1 mmol) substituted ketone was added (1 mmol), and sodium hydroxide (5%) as base and methanol (10 ml) as a solvent. The reaction mixture was stirred at room temperature for 4 h. After the completion of the reaction (TLC), the reaction mixture was poured on ice cold water and filtered. The solid was washed with water and dried to afford the crude product. The crude product was recrystallized from methanol to get pure compound. The synthesized chalcones have been enlisted in figure 5.1.1 (Joshi et al., 2016)

Br O_2N O **1a**

O O O_2N O **1b**

O I O_2N O **1c**

O O O O_2N O **1d**

O O_2N O **1e**

Figure 5.1.1: List of chalcones synthesized by base catalyzed Claisen- Schmidt Condensation.

5.1.1.1 Synthesis of (E)-**3-(2-nitrophenyl)-1-(3, 4, 5-trimethoxyphenyl) prop-2-en-1-one (1d)**

Yield- 75.3%, Brownish solid, m.p. 138-140 ^{o}C

Mass (EI): m/z: 343 (M^{+})

IR (KBr, cm^{-1}): 1669(C=O), 1130(C-O stretch), 1534 and 1346 (NO_2 stretch)

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: 8.10 (1H,d,J=12Hz), 8.08 (1H,d, J=8Hz) 7.74 (1H,t,J=12Hz), 7.71 (1H,t,J=8Hz), 7.59 (1H,d,J=8Hz), 7.29 (2H,s), 7.22 (1H,d,J =12Hz) , 3.98 (6H,s), 3.94 (3H,s)

5.1.2 General procedure for the synthesis of pyrazolines (2a-2e)

A mixture of chalcone (1 mmol) and phenyl hydrazine hydrate hydrochloride (3 mmol) was refluxed in methanol for 2h. After completion of reaction (TLC), the crystallized product was collected by decanting extra methanol from the reaction mixture and used for next step without further purification. The synthesized pyrazolines have been enlisted in figure 5.1.2 (Joshi et al., 2016).

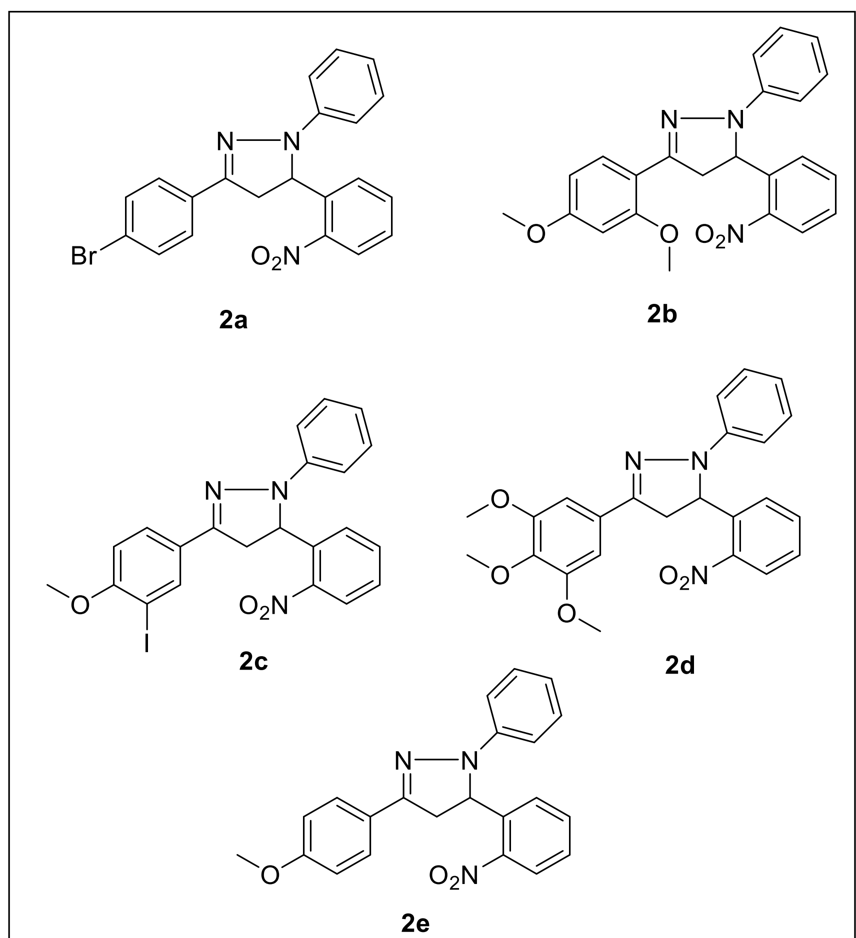
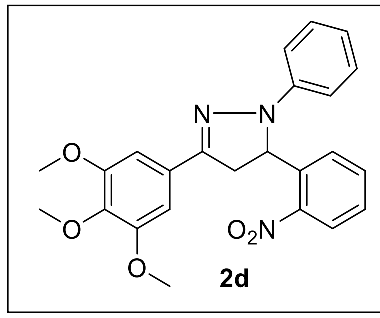


Figure 5.1.2: List of pyrazolines synthesized by Michael addition.

5.1.2.1 Synthesis of 5-(2-nitrophenyl)-1-phenyl-3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazole (2d)



Yield- 80%, orange crystalline solid,

Mass (EI): m/z: 433 (M^+)

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: 8.15 (1H, d, J=7.96Hz), 7.67 (1H, t, J=7.32 Hz), 7.55(1H, t, J=7.2 Hz), 7.28 (1H, d, J=7.72 Hz), 7.15(2H, m,), 7.01

(2H, s), 6.94 (2H, d, J=8.04 Hz), 6.73(1H, t,J=7.28 Hz), 5.92(1H, dd, J_{12}=5.7 Hz ,J_{34} =5.8 Hz), 4.11(2H, dd, J_{12}= 12.43 Hz,J_{34} =12.44 Hz), 3.84(6H ,s),3.69 (3H,s)

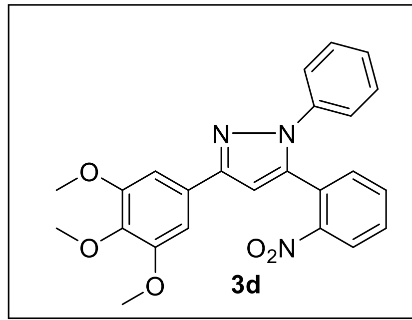
^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ:153.4, 147.9,147.6,144.0 ,138.8,137.0 ,134.8,129.3,128.0,125.8,119.2,113.0,103.5,79.3,60.53,56.32,43.12

5.1.3 General procedure for the synthesis of pyrazoles (3a-3e)

A mixture of pyrazoline (1 mmol) was refluxed with catalytic amount of molecular iodine in DMSO at 60-80 ^{0}C for 4 h. After the completion of reaction (TLC), the reaction mixture was poured in ice cold water, product was extracted using ethyl acetate. Organic layer was washed with sodium thiosulphate to remove trace of iodine. Finally the organic layer was filtered, dried over sodium sulphate and was finally evaporated under reduced pressure using rotary evaporator to obtain the product which was used for next step without further purification. The synthesized pyrazolines have been enlisted in figure 5.1.3

Figure 5.1.3: List of pyrazoles synthesized by oxidation.

5.1.3.1 Synthesis of 5-(2-nitrophenyl)-1-phenyl-3-(3,4,5-trimethoxyphenyl)-1H-pyrazole(3d)



Yield- 72%, orange crystalline solid,

Mass (EI): m/z: 431 (M^+)

IR (KBr, cm^{-1}):1526 and1346 (NO_2 stretch)

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: 8.05 (1H, d, J=8Hz), 7.81 (2H, t, J=8 Hz) 7.73(1H, d, J=4 Hz), 7.70 (1H, t, J=8 Hz), 7.68(2H,t, J=4 Hz), 7.64 (1H, d, , J=8Hz), 7.19 (1H, s), 7.15 (2H, s), 7.02 (2H, d, J=8 Hz), 3.81(6H,s) ,3.66(3H,s)

^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ: 153.76 ,152.01,148.61 ,139.84,139.11, 138.51,138.13,134.53,133.34,131.58,128.33,126.40,125.39,125.03,107.34,103.28,93.94,60.64,56.46

5.1.4 General procedure for the synthesis of proposed compounds (4a-4e)

A pyrazole (1g) was dissolved in methanol and refluxed with 5-8 equivalent of iron metal and few drops of HCl at 70^0C for 4 h. After the completion of the (TLC), the reaction mixture passed through celite for the removal of metal after the methanol was evaporated under reduced pressure using rotary evaporated followed by the neutralized of the reaction mixture using 5% NaOH solution. Solid product was then extracted with ethyl acetate. Organic layer was washed with brine, dried over sodium sulphate and was evaporated under pressure using rotary evaporator to obtain the product. further product was purified using column chromatography (silica gel 60-120; 5% ethyl acetate 95%petroleum ether) The final compounds was further characterized by melting point, mass, IR and NMR spectroscopy. The synthesis of proposed compounds as shown in figure 5.1.4

Figure 5.1.4: List of Synthesized of compounds.

5.1.4.1. Synthesis of 2-(3-(4-bromophenyl)-1-phenyl-1H-pyrazol-5-yl) aniline (4a)

Yield: 72%, Brownish crystalline solid, m.p. 115-117 ^{o}C

Mass (EI): m/z: 389 (M^{+})

IR (KBr, cm^{-1}): 3451 (NH_2) stretch, 1527 (C=N), 1619 (NH_2) bend, 700(C-Br)

UV (MeOH): λ_{max} at 205 nm and 264 nm

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0)δ: 7.82(2H,d,J=8Hz),7.58(2H,d,J=8 Hz), 7.42(2H,d,J=8Hz),7.35-7.31(3H,t,J=16Hz),7.22-7.18(1H,t,J=16Hz), 7.00 (1H,d , J=4 Hz) ,6.83(1H,s) ,6.76-6.72(2H,t,J=16 Hz),3.79(2H,s)

^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ: 151.03, 144.64, 141.29, 139.82, 131.89, 131.10, 130.24, 128.86, 127.29, 127.22, 124.66, 123.79, 121.97, 118.29, 115.79, 115.66, 105.89

5.1.4.2. Synthesis of 2-(3-(2,4-dimethoxyphenyl)-1-phenyl-1H-pyrazol-5-yl)aniline (4b)

Yield: 60%, Yellowish crystalline, m.p. 124-126 °C

Mass (EI): m/z: 371 (M^+) 371(Base peak)

IR (KBr, cm^{-1}): 3386(NH_2) stretch, 1525(NH_2) bend, 1599(C=N)

UV (MeOH): λ_{max} at 215 nm and 305 nm

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ:8.09-8.07(1H,d,J=8 Hz),7.44 7.42(1H,d,J=8Hz),7.31-7.28(2H,d,J=12Hz),7.26(1H,s),7.24-7.22(1H,d,J=8 Hz),7.20-7.16(1H,t,J=16Hz),7.05-7.01(2H,tJ=16Hz)6.75-6.71(2H,t,J=16 Hz),6.63-6.61(1H,d,J=8 Hz) ,6.59(1H,s),3.89(3H,s),3.92(3H,s)

5.1.4.3. Synthesis of 2-(3-(3-iodo-4-methoxyphenyl)-1-phenyl-1H-pyrazol-5-yl) aniline (4c)

Yield: 67%, yellow solid, m.p. 125-127 ^{o}C

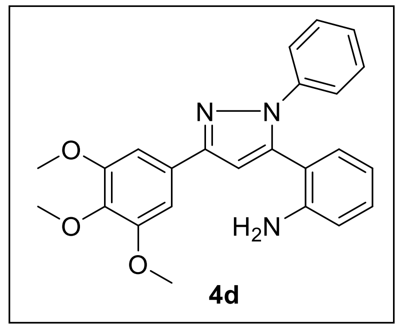
Mass (EI): m/z: 467 (M^+)

UV (MeOH): λ_{max} at 216 nm and 284 nm

IR (KBr, cm^{-1}): 3458 (NH_2) stretch, 1530 (NH_2) bend, 1130(C-O), 750(C-I)

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: ^{1}H NMR (400 MHz, CDCl3, TMS = 0) δ:8.38(1H,dd,J_{12}=4,J_{14}=16Hz)7.88(1H,d,J=8Hz),7.41(1H,d,J=8 Hz), 7.35-7.25 (5H,m),7.20(1H,t,J=12Hz),7.00-6.98(1H,dd,J=8Hz),6.91(1H,d,J=12Hz), 6.77(1H,s),6.72(1H,t,J=8 Hz) ,3.95(2H,s)

5.1.4.4. Synthesis of 2-(1-phenyl-3-(3,4,5-trimethoxyphenyl)-1H-pyrazol-5-yl)aniline (4d)



4d

Yield: 65%, yellow solid, m.p. 120-122 ^{o}C

Mass (EI): m/z: 401 (M^+)

IR (KBr, cm^{-1}): 3055 (NH_2) stretch, 1586 (NH_2) bend, 1645(C=N)

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: 6.75(1H, t, J=8 Hz), 7.49(1H,d, J=8 Hz),7.44(1H,d,J=8 Hz),7.33(1H, t, J=8 Hz),7.31(2H,s),7.18(2H, t, J=8

Hz),7.14(1H, t, J=4Hz),7.04(1H,dd,J_{12}=8 Hz,J_{34}=8 Hz),6.74(2H, d, J=8 Hz),3.97 (6H,s),3.92(3H,s)

5.1.4.5. Synthesis of 2-(3-(4-methoxyphenyl)-1-phenyl-1H-pyrazol-5-yl) aniline (4e)

Yield: 72%, yellow solid, m.p. 126-128 ^{o}C

Mass (EI): m/z: 341 (M^{+})

IR (KBr, cm^{-1}): 3061 (NH_2) stretch, 1528 (NH_2) bend

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: 8.02(1H, d, J=8 Hz), 7.81(2H,d, J=8 Hz) 7.64(1H,t,J=8 Hz),7.62(1H, d, J=8 Hz),7.26(1H,S),7.18(1H, t, J=12Hz),7.11(2H, d,J=12Hz),7.02(2H,t,J=12Hz),6.91(1H, t, J=12 Hz),6.80(2H,dJ=8 Hz,),3.77(3H,s)

5.2 Biology

5.2.1 Chemicals

- The Cancer cell lines were cultured with media – DMEM (Dulbecco's Modified Eagle's Medium), fetal bovine serum (FBS) were purchased from HiMedia Penicillin/Streptomycin antibiotic solution and Phosphate buffer saline.
- **MTT** dye used for MTT assay was purchased from HiMedia
- **DMSO,** extrapure AR was purchased from SRL

5.2.2 Instruments

The experiment involving cell culture was carried out under aseptic conditions via 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 used to observe cancer cells. Further the absorption was measured by using spectrophotometer.

Table 5.2 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

5.2.3 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 a 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.

5.2.4 Routine assay in culture laboratory

A. Culturing of cell lines

DMEM is used as a medium for culturing of the cancer cell lines as it is adherent cells, 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 and supernatant was removed and pellet was resuspended in media (2 ml). With automated cell counter, cell number was counted. The cells were moved to fresh media every two days.

B.Maintenance and sub-culturing of cell lines

The 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% CO2.

Further sub-culturing of cells was done in 25 cm^2 flasks up to when 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. The 1 ml of media containing serum was added after 5 minutes for stopping the action of trypsin. Then, the cells were transferred to 15 ml centrifuged 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).

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

The first MTT assay was specifies by Mosmann for the biological activity. It is also called cell viability assay(Mosmann, 1983). It is an in vitro colorimetric assay used for the measurement of cell proliferation. The tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) when start in cells is reduced to an insoluble coloured formazan product by mitrochondrial succinate dehydrogenase and when it passes to the mitochondria it gets solubilized with DMSO.

Figure 5.2 Reduction of MTT

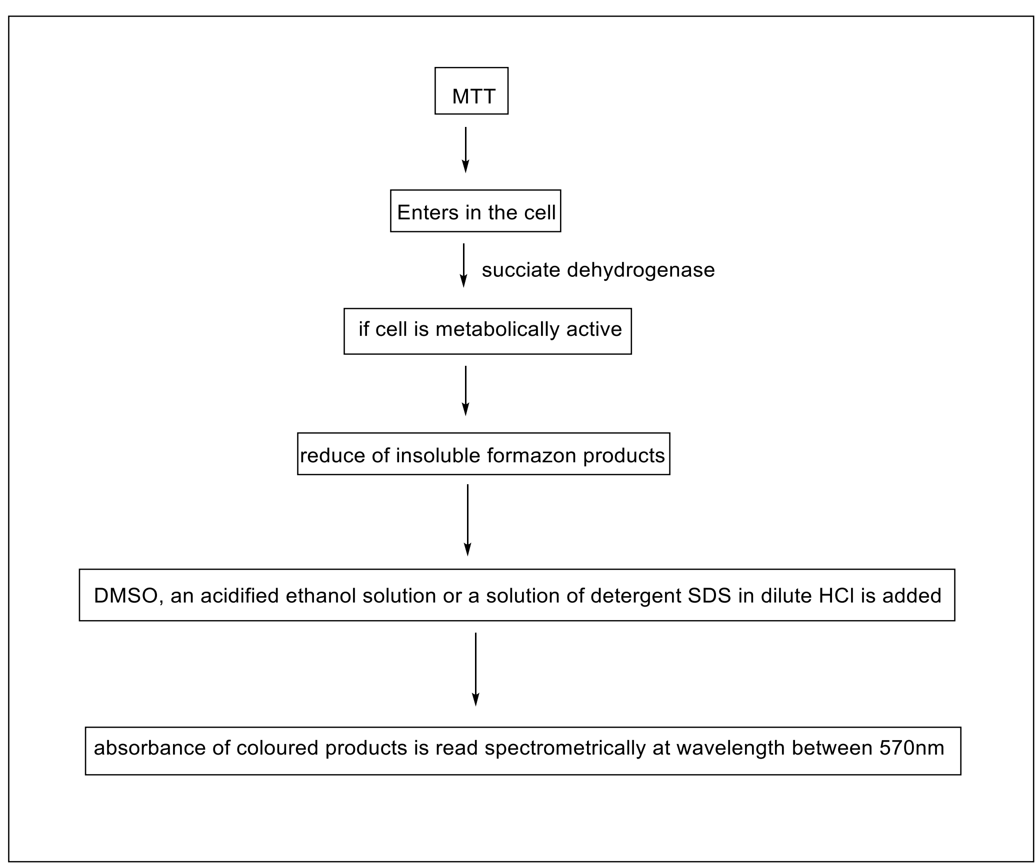


Figure 5.3 Basic principal of MTT assay

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

Procedure: The cell 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)

5.3 Molecular docking:

The htopoI and htopoII complexed with Camptothecin (1T8I) and doxorubicin (1ZXM). The ligand were docked with the active site of htopoI/II with the help of

Maestro11.1. It is a site of automated docking that designed how small molecules bind to receptor. For example substrate bind to receptor of 3D structure. By the use of Maestro 11.1 protein preparation module, the preparation of protein structure initially processed by the missing side chain and bonds. The extra water molecules were removed and protonation was done. After this in the active site protein grid was generated and docking score were evaluated.

CHAPTER-6
RESULTS AND Discussion

6. Results and discussion

6.1 Synthesis

For the synthesis of target compounds, we proposed the route (scheme 6.1)

5 + 6 →(a) 1a-1d →(b) 2a-2d →(c) 3a-3d →(d) 4a-4b

a: Claisen-Schmidt condensation;

b: Michael addition

c: oxidation

d: reduction

Reagents and conditions (a) 5%, NaOH,Methanol, 4-5h (b)phenyl hydrazine, MeOH,rt (c)I_2 ,DMSO,reflux,4h (d)Fe,HCl,MeOH,rt,4h

Scheme 6.1: Proposed route for the synthesis of target compounds

6.1.1 Synthesis of chalcones

The synthesis of chalcone derivatives was carried out through base catalyzed Claisen-Schmidt condensation. Mechanistically, in base catalyzed reaction, the base abstracts proton from the α-carbon which leads to the formation of enolate ion. In next step the attack of nucleophile (carbanion) on the carbocationic center of aldehyde occurs, followed by the dehydration of water or an alcohol molecule (figure 6.1.1)

6.1.1 General mechanism of Claisen-Schmidt condensation.

6.1.1 Synthesis of (E)-3-(2-nitrophenyl)-1-(3, 4, 5-trimethoxyphenyl) prop-2-en-1-one (1d)

The synthesis of **1d** was carried out through Claisen-Schmidt condensation of 2-nitrobenzaldehyde (5) with 3, 4, 5 trimethoxyacetophenone (6) aryl ketones in the presence of methanol and few drops of NaOH (scheme 6.1.1)

Scheme 6.1.1: Synthesis of (E)-3-(2-nitrophenyl)-1-(3, 4, 5-trimethoxyphenyl) prop-2-en-1-one **(1d)**

1d was characterized by melting point (m.p), mass and NMR spectroscopic techniques. In the mass spectrum, molecular ion peak was observed at m/z = 343. The fragmentation pattern showed initial loss of nitro group with the peak at m/z=297. As shown in figure 6.1(a)

m/z=343 m/z=297

Figure 6.1(a): fragmentation of **1d**

Characteristic IR absorption frequencies at 1669 cm^{-1} for carbonyl group which was much lower than the normal value of 1710 cm^{-1}. This is because due to decrease α,β-double bond conjugation with the carbonyl group. The conjugation were increase the single bond character of the (C=O) in the resonance hybrid.

In ^{1}H NMR spectrum peaks both α and β proton show the coupling constant J=12Hz indicating the formation of trans (E) compound.

6.1.2 Synthesis of 5-(2-nitrophenyl)-1-phenyl-3-(3,4,5-trimethoxyphenyl)-1H-pyrazole (2d)

In next step, synthesis of **2d** was carried out by refluxing mixture of 1d and phenyl hydrazine (1:3 eq) for 4 h. The pure product crystallized in the reaction mixture which was filtered, dried and was used for next step after characterization (scheme 6.1.2)

(*E*)-3-(2-nitrophenyl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one + phenylhydrazine hydrochloride ($NH.NH_2.HCl$) → (MeOH, rt,4h) → **2d**

Scheme 6.1.2 Synthesis of 5-(2-nitrophenyl)-1-phenyl-3-(3,4,5-trimethoxyphenyl) -1H-pyrazole **(2d)**

2d was characterized by mass, NMR and ^{13}C spectroscopic techniques. In ^{1}H NMR spectrum of the **2d** showed the ABX system with two double doublets (5.92 ppm and 4.11 ppm) out of which one is double doublet was deshileded and appeared more downfield than other. The two double doublets represent that the two proton of the pyrazoline nucleus were confirmed.

^{13}C spectrum showed a characteristic peak at 43.12 ppm at C_3 confirmed the formation of pyrazoline **2d**

In the mass spectrum, molecular ion peak was observed at m/z =433.

6.1.3 Synthesis of 5-(2-nitrophenyl)-1-phenyl-3-(3,4,5-trimethoxyphenyl)-1H-pyrazole(3d)

Further, synthesis of 3d was carried out by refluxing mixture of 2d and iodine and DMSO for 4h. The pure product crystallized in the reaction mixture which was filtered, dried and was used for next step after characterization. (Scheme 6.1.3)

2d → (I_2 DMSO, reflux 4h) → **3d**

Scheme 6.1.3: Synthesis of 5-(2-nitrophenyl)-1-phenyl-3-(3,4,5-trimethoxy phenyl) -1H-pyrazole(3d)

3d was characterized by mass, NMR and ^{13}C spectroscopic techniques. In ^{1}H NMR spectrum of the **3d** characteristic singlet at 7.19 ppm and also, the absence of characteristic ABX pattern of pyrazoline confirmed the formation of pyrazole.

^{13}C spectrum show the presence of characteristic peak at 103.28 ppm for pyrazole which was at 43.12 ppm in pyrazoline.

In the mass spectrum, molecular ion peak was observed at m/z =431

6.1.4 Final compounds (4a-4e)

While it was planned to reduce the nitro group of **(3a-3e)** under basic condition, Fe/HCl and MeOH. The reaction was heated 70°C. The TLC showed two spots. Upon isolation through column chromatography (Silica gel # 60:120; EtOAc: Pet Ether :: 50:50).

6.1.5.1 Synthesis of 2-(3-(4-bromophenyl)-1-phenyl-1H-pyrazol-5-yl) aniline (4a)

Fe/HCl,MeOH
reflux,4h

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

4a
2-(3-(4-bromophenyl)-1-phenyl-1*H*-pyrazol-5-yl)aniline

In the mass spectrum molecular ion peak was observed at m/z= 389. Also, characteristic Br pattern (1:0.9) peak were observed.

IR spectrum showed the characteristic peak of primary amine stretching and bending at 3451 cm^{-1} and 1619 cm^{-1} respectively and bromine at 700 cm^{-1}

The proton NMR spectrum showed a characteristic doublet of two proton of ring A at 7.84 ppm due to Br which is more deshileded than NH_2 .The NH_2 showed singlet of two proton at 3.79 ppm which indicate the presence of NH_2 at ring B.

UV spectra characterized by the presence of absorbance, the high energy band (260-264nm) was due to π- π* transition.

6.1.5.2 Synthesis of 2-(3-(3, 4-dimethoxyphenyl)-1-phenyl-1H-pyrazol-5-yl) aniline (4b)

Fe/HCl,MeOH

reflux,4h

3b

3-(3,4-dimethoxyphenyl)-5-(2-nitrophenyl)-1-phenyl-1*H*-pyrazole

4b

2-(3-(3,4-dimethoxyphenyl)-1-phenyl-1*H*-pyrazol-5-yl)aniline

The mass spectrum showed a peak at m/z 371 as a base peak

IR spectrum showed the characteristic peak of primary amine stretching and bending at 3386 cm^{-1} and 1525 cm^{-1}, respectively.

The proton NMR spectrum showed a characteristic singlet of six proton of OCH_3 at 3.92 ppm and 3.89 ppm. The pyrazole aromatic proton appeared as singlet at 6.59 ppm.

UV spectra characterized by the presence of two absorbance, the lower energy band (300-305nm) was due to n- π* transition and the higher energy band (205-215nm) was due to π- π* transition.

6.1.5.3. Synthesis of 3 2-(3-(3-iodo-4-methoxyphenyl)-1-phenyl-1H-pyrazol-5-yl) aniline (4c)

Fe/HCl,MeOH

reflux,4h

3c

3-(3-iodo-4-methoxyphenyl)-5-(2-nitrophenyl)-1-phenyl-1*H*-pyrazole

4c

2-(3-(3-iodo-4-methoxyphenyl)-1-phenyl-1*H*-pyrazol-5-yl)aniline

The mass spectrum showed a peak at m/z 467 as a base peak.

IR spectrum showed the characteristic peak of primary amine stretching and bending at 3458 cm^{-1} and 1530 cm^{-1}.respectively.

The proton NMR spectrum showed a characteristic singlet of three proton of OCH_3 at 3.95ppm. The pyrazole aromatic proton appeared singlet at 6.77 ppm.

6.1.5.4 Synthesis of 2-(1-phenyl-3-(3,4,5-trimethoxyphenyl)-1H-pyrazol-5-yl)aniline(4d)

Fe/HCl,MeOH

reflux,4h

3d

5-(2-nitrophenyl)-1-phenyl-3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazole

4d

2-(1-phenyl-3-(3,4,5-trimethoxyphenyl)-1*H*-pyrazol-5-yl)aniline

The mass spectrum showed a peak at m/z 401

IR spectrum showed the characteristic peak of primary amine stretching and bending at 3055 cm^{-1} and 1586 cm^{-1}, respectively.

The proton NMR spectrum showed a characteristic singlet of three proton of OCH_3 at 3.92ppm.

6.1.5.5 Synthesis of 2-(3-(4-methoxyphenyl)-1-phenyl-1H-pyrazol-5-yl) aniline (4e)

Fe/HCl,MeOH

reflux,4h

3e

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

4e

2-(3-(4-methoxyphenyl)-1-phenyl-1*H*-pyrazol-5-yl)aniline

The mass spectrum showed a peak at m/z 341

IR spectrum showed the characteristic peak of primary amine stretching and bending at 3061 cm^{-1} and 1528 cm^{-1}, respectively.

The proton NMR spectrum showed a characteristic singlet of three proton of OCH_3 at 3.77 ppm. The pyrazole show singlet at 7.26 ppm.

6.2 Biological studies

6.2.1 Evaluation of anticancer properties of the compound

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

6.2.2 Antiproliferative activity against MDA-MB 231cell line

All the synthesized four compounds (**4a, 4b, 4c, 4d** and **4e**) were evaluated for the antiproliferative activity against MDA-MB-231 (Breast) Cancer Cell line.
Among them **4b** and **4c** exhibited good inhibitory potential, around 40% both at a very low concentration of 1 µM. Further at concentration of 5 µM, **4a** and **4b** displayed 80% and 75% inhibition.

4b **4c**

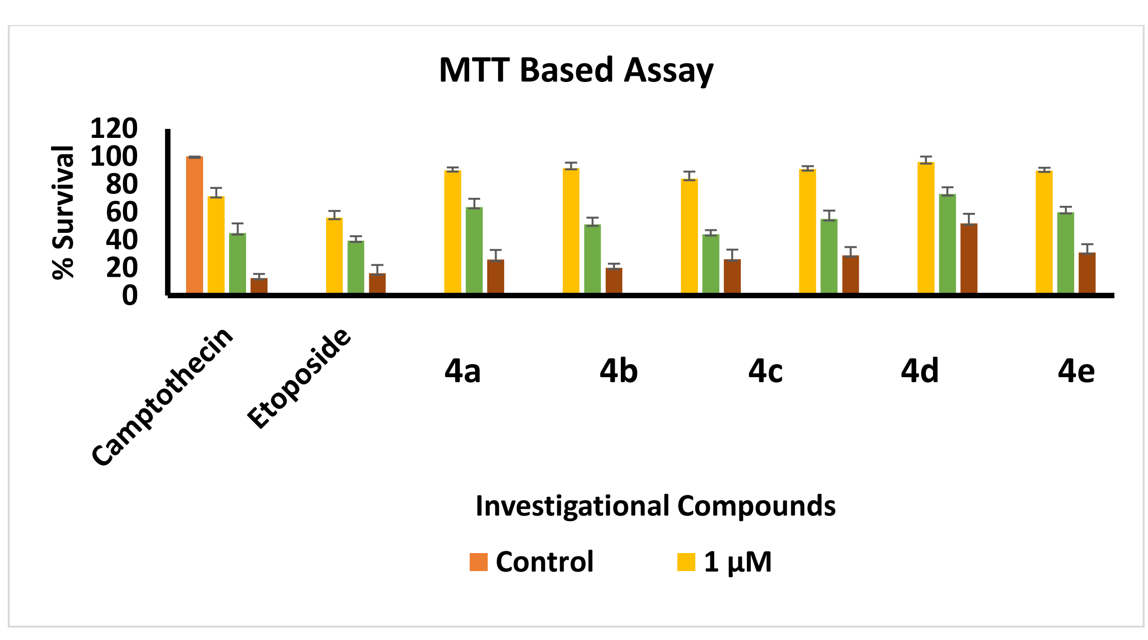


Figure 6.2.2 Percent inhibition of breast cancer cell line MDA-MB 231 in response to treatment with synthesized compounds at concentrations of 1 µM, 5 µM and 25 µM for time duration of 48 h. Data is expressed as mean values ±S.D. of three independent experiments.

IC_{50} Value: IC_{50} is the inhibitory concentration at which 50% of the target is inhibited**.** It is a measure of the effectiveness of a drug in inhibiting a special biological function

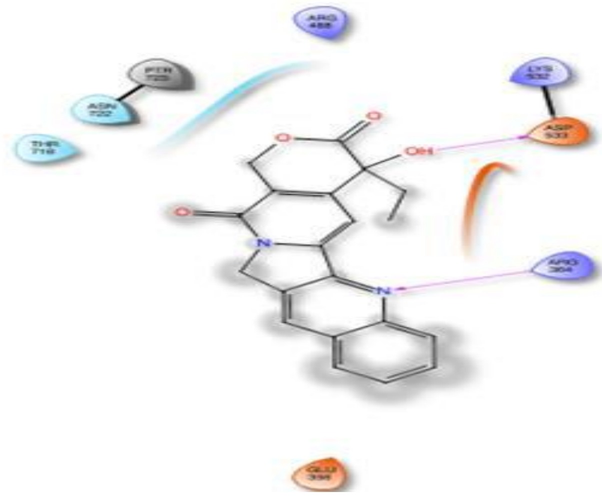
Table 6.2 IC_{50} values of synthesized compounds against MDA-MB 231cell line

S.no	Investigation compound	IC_{50} (µM) $\pm$ SD
1.	Camptothecin	4.16
2.	Etoposide	2.37
3.	4a	12.18
4.	4b	4.00
5.	4c	5.56
6.	4d	11.83
7.	4e	> 25

6.3 Molecular modeling studies

The molecular modeling studies of designed compounds were performed using Maestro 11.0 it shows the interaction between small molecules (ligands) and macromolecules target (receptor) and finally to the binding capacity. The compounds were docked in the active site to identify the interaction of topo I. its resolution capacity is 3.0 Å and having 592 amino acid residues. The suitable interaction between ligand and receptor were scored (module of Schrödinger Suite). Confirmation of the docking protocol was done by re-docking the co-crystallized topotecan (camptothecin) analogue to the DNA in the Topo I.

From 2D interaction diagrams it was found that standard inhibitor camptothecin binds to the target site and showed hydrogen bond interactions with ASP 533. Doxorubicin presented hydrogen bond interactions with ARG 98. Molecular docking studies of designed compounds demonstrated that the designed compounds have similar hydrogen bonding interactions with several protein bases as of standard compounds and π- π interaction. The compounds **4a**, **4b**, **4c**, **4d**, and **4e** have shown the corresponding amine group and aromatic interactions with various amino acids present in the binding cavity. The in silico results have corresponded to the in vitro results revealing the importance of free NH_2. Among all the compounds **4b** have shown better dock score results in comparison to standard molecules as shown in table 6.3



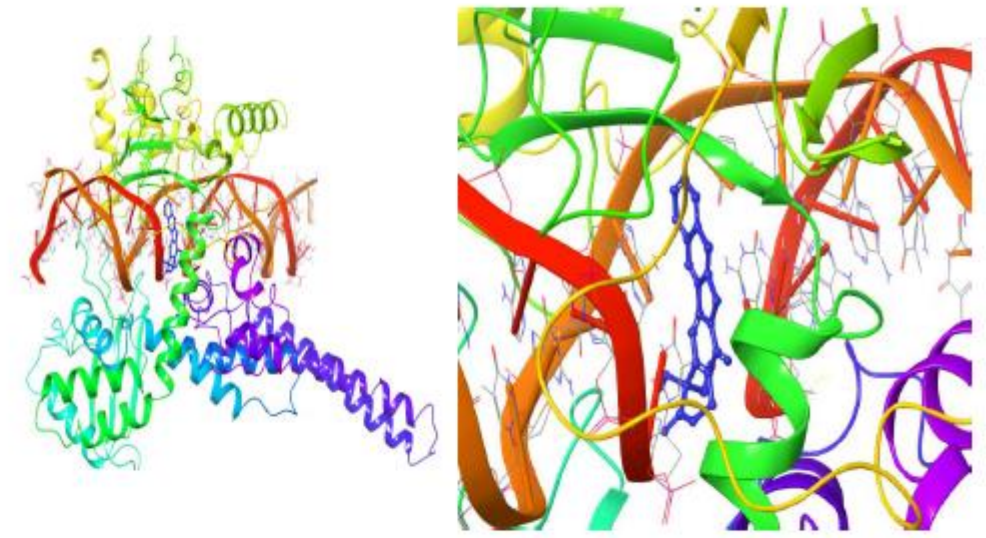


Figure 6.3(a): 2D and 3D interaction of camptothecin

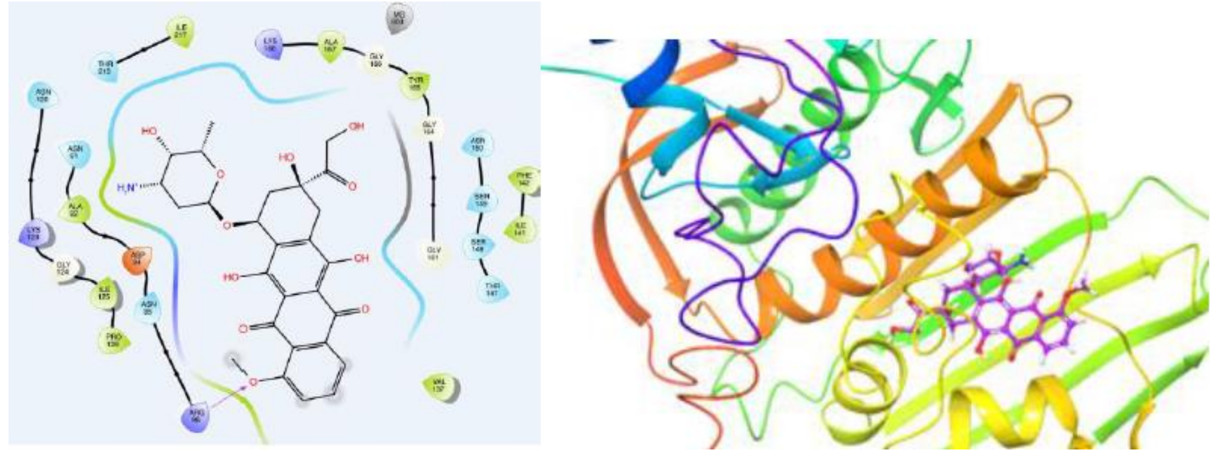


Figure 6.3(b):2D and 3D interaction of Doxorubicin

Table 6.3 Docking studies of standard, **Camptothecin (htopoI)**, **doxorubicin (htopoII)** and synthesized compounds.

Docking in htopoI(1T8I)

Structure (Name)	Docking pose	Dock score
Camptothecin 1T8I		- 8.829kcal/mol
4b		--7.4kcal/mol

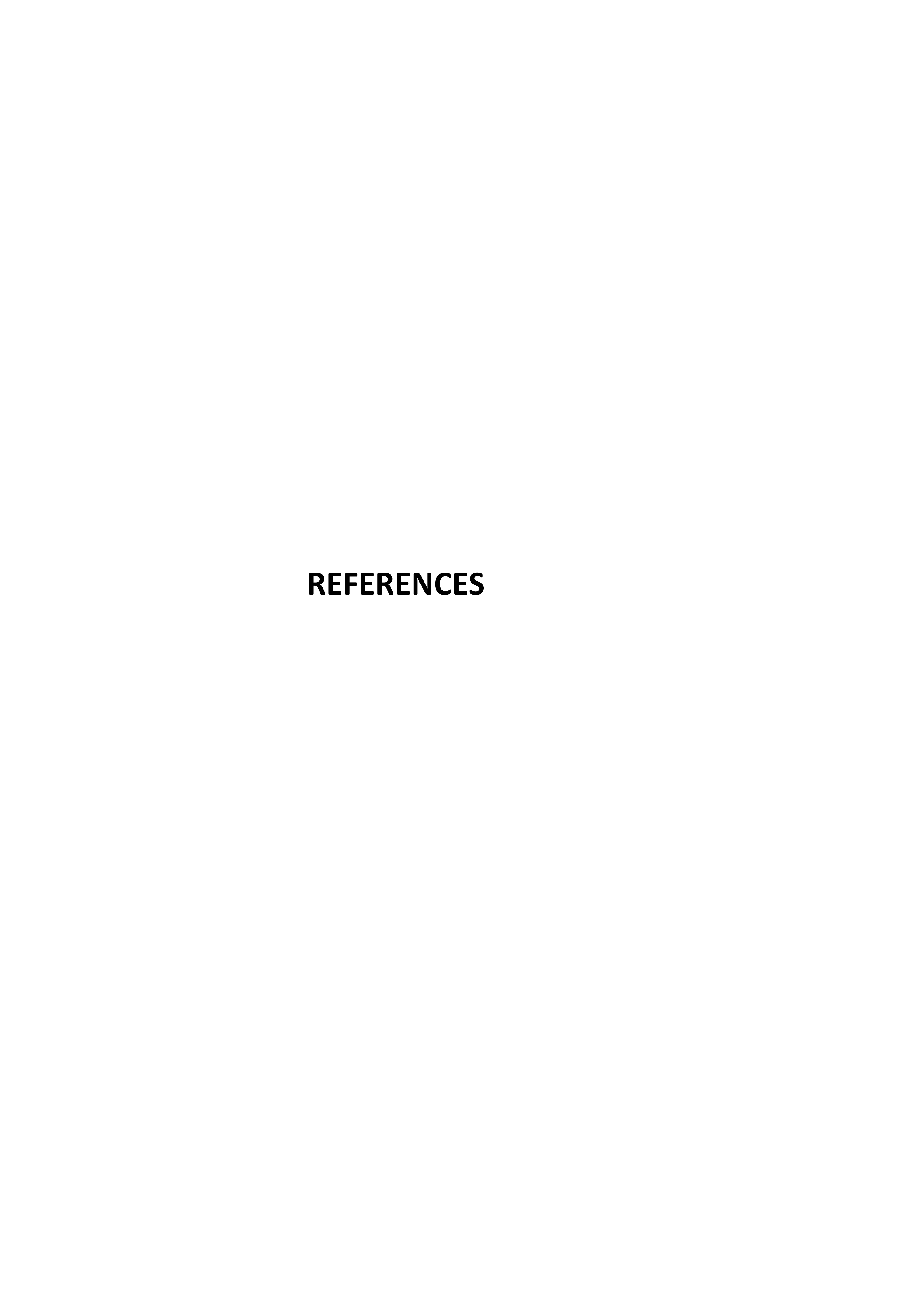
Docking in htopoII (1ZXM)

Structure (Name)	Docking pose	Dock score
Doxorubicin		-4.641 kcal/mol
4b		-6.6 kcal/mol

CHAPTER-7
CONCLUSIONS

7. Conclusions

Pyrazole based compounds were rationally designed as dual topo-I/II inhibitors. An easily accessible synthetic protocol was developed for the synthesis of target compounds which were further evaluated and explored for their antiproliferative activity on breast cancer cell line (MDA-MB 231) by performing MTT assay. Among which one compound, **4b** with IC_{50} value of 4.00 μM, was found to show comparable antiproliferative activity with camptothecin but lesser than etoposide. The synthesized compounds can be further explored for lead optimization, generation of library compounds, screening them against various cell lines, drawing their structure activity relationships and hence developing a promising drug.



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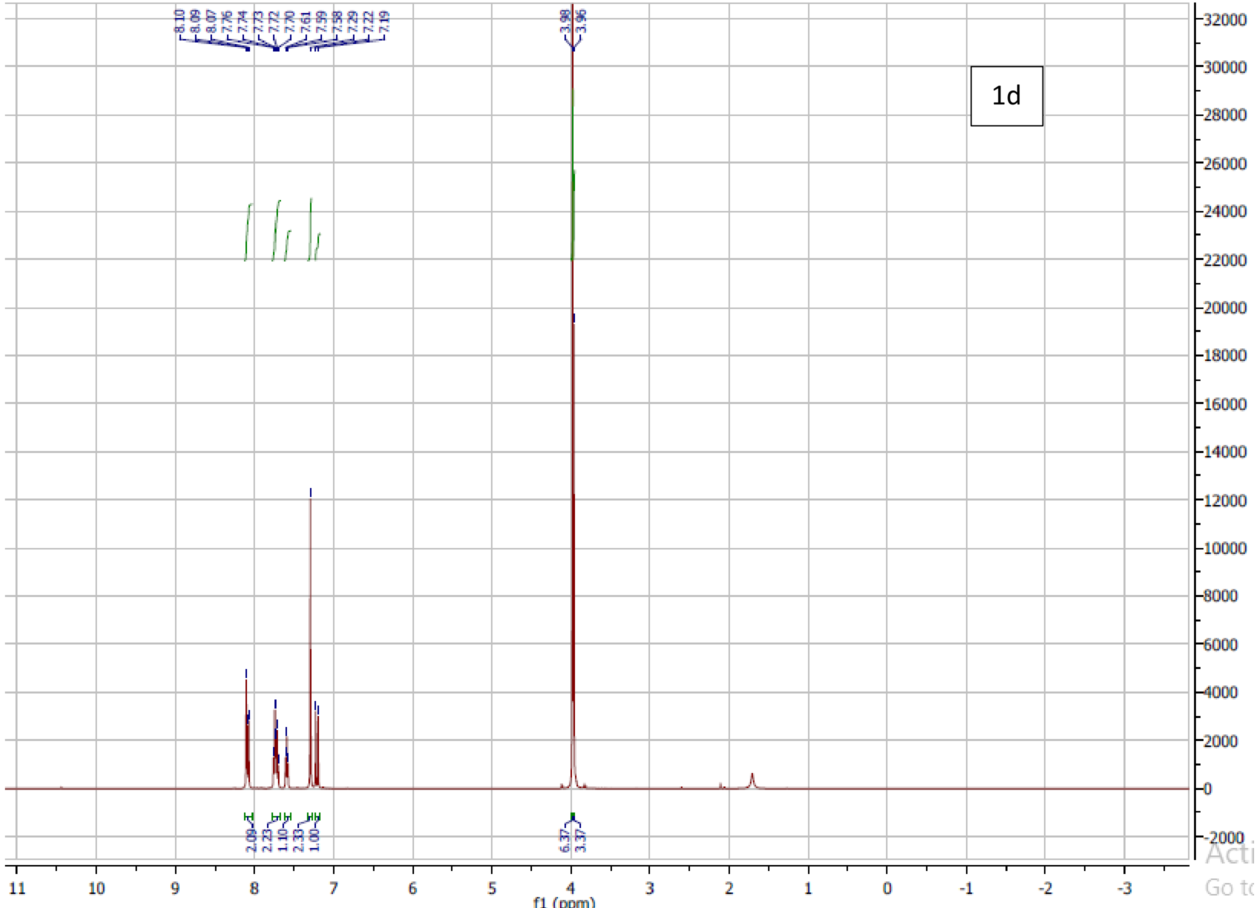
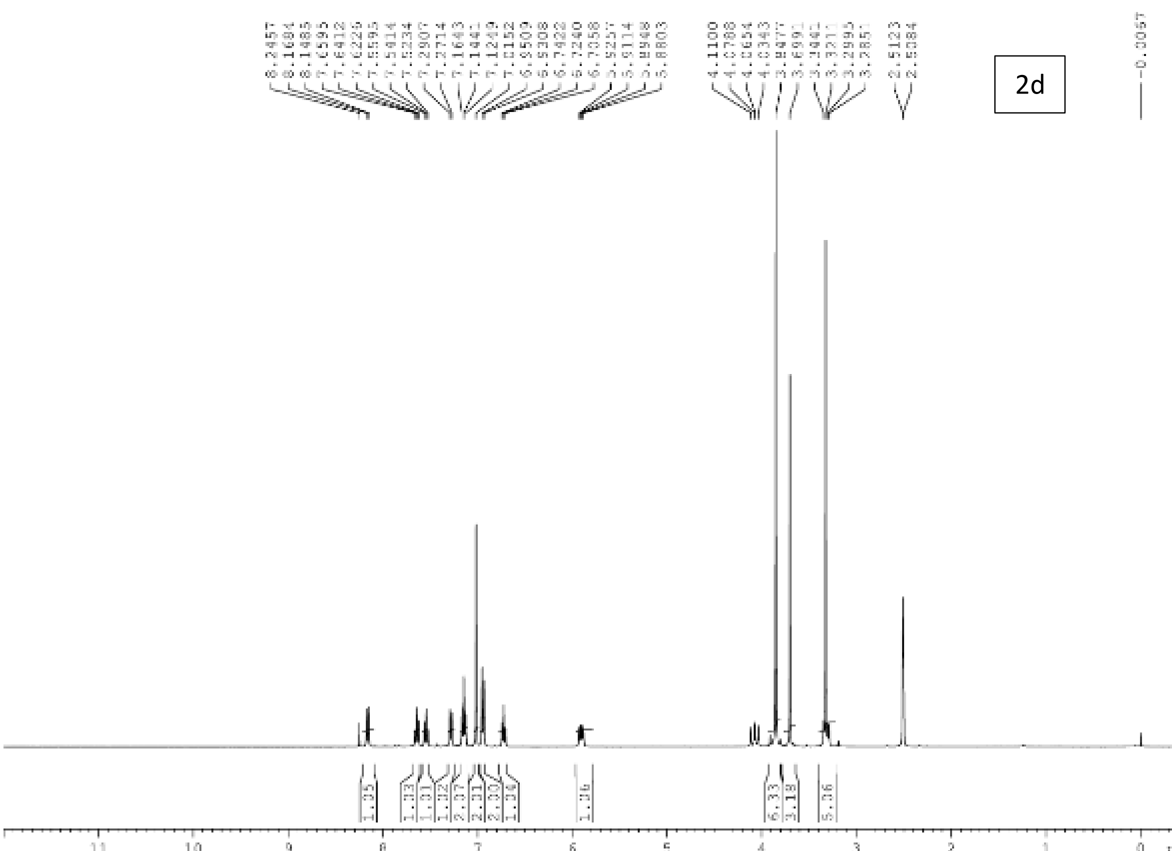
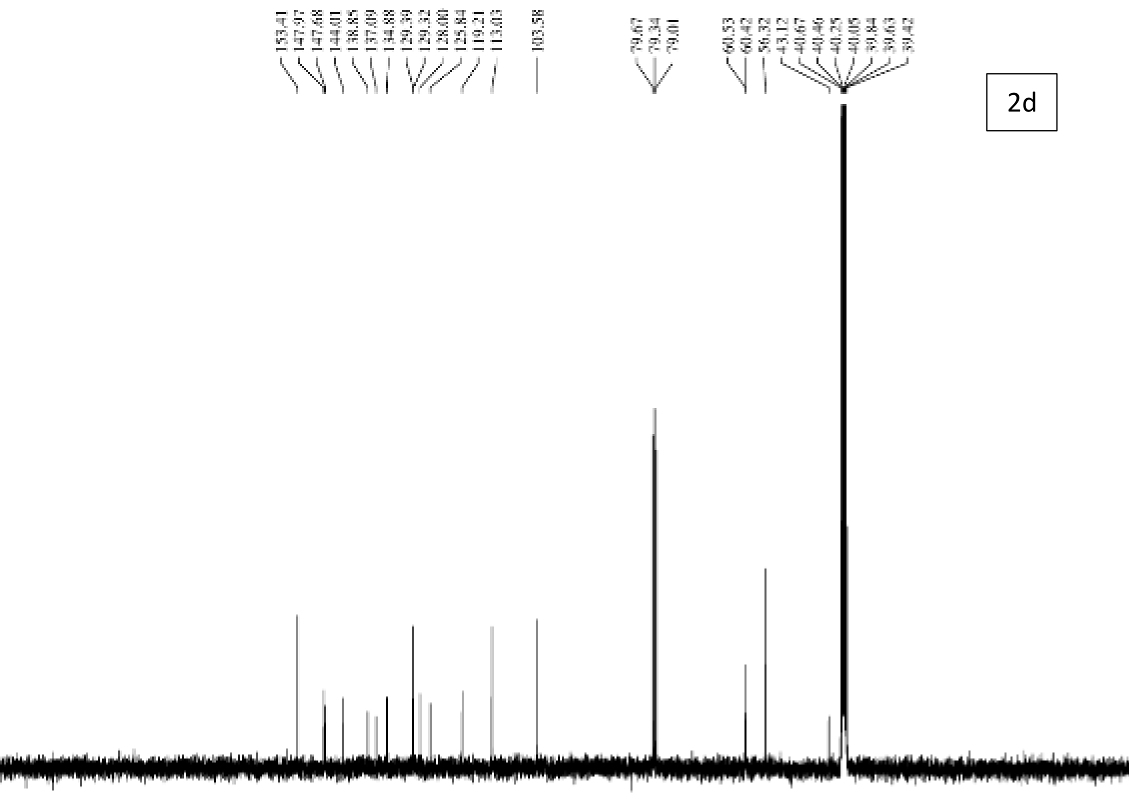
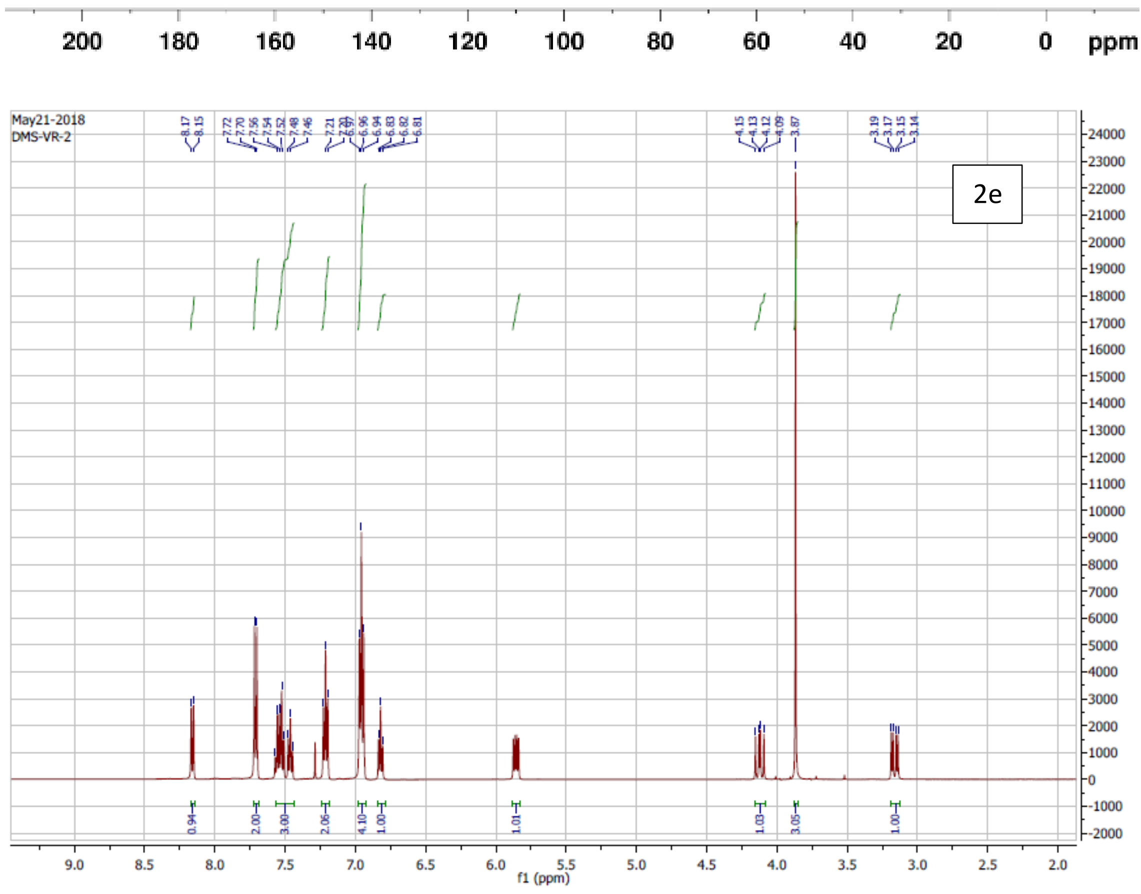
APPENDIX
Spectra of Representative Compounds

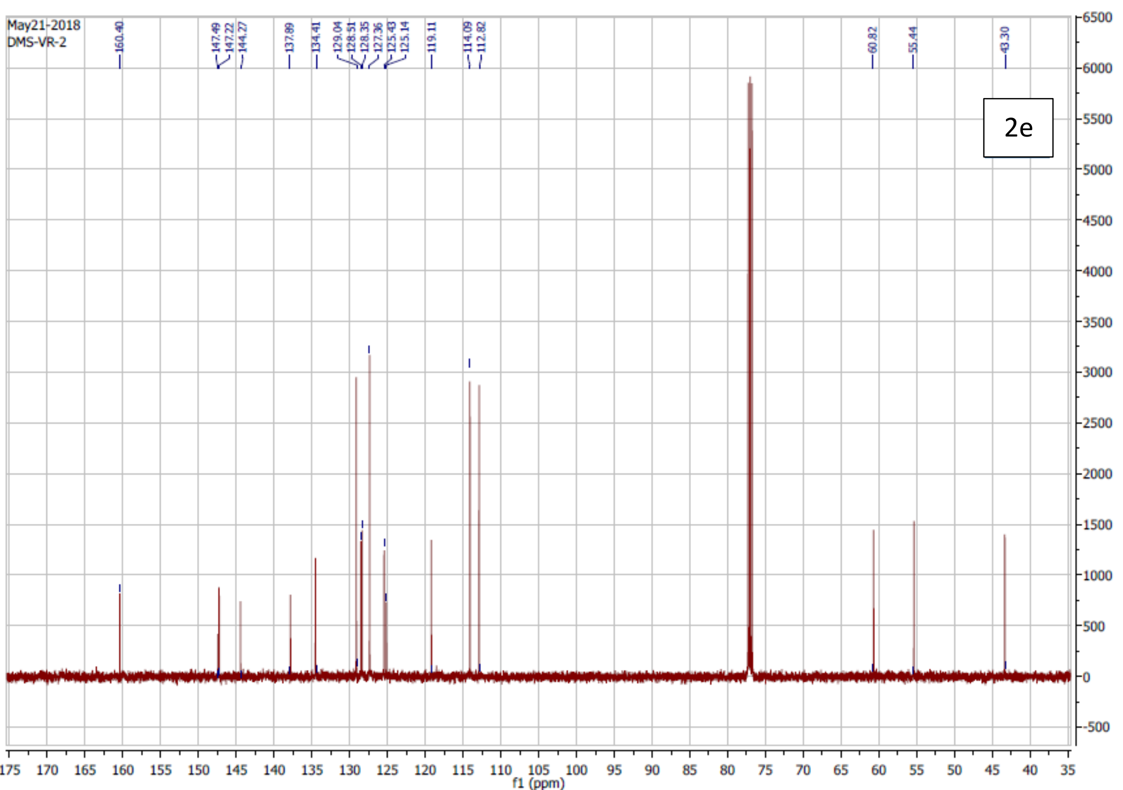


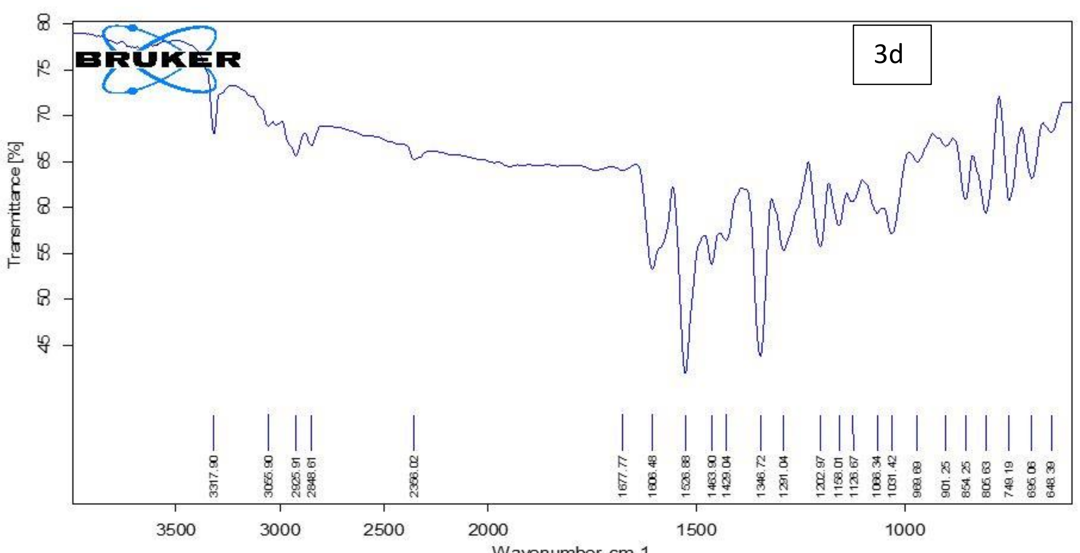
8.10
8.09
8.07
7.76
7.74
7.73
7.72
7.70
7.61
7.59
7.58
7.29
7.22
7.19
3.98
3.96
1d
2.09
2.23
1.10
2.33
1.00
6.37
3.37
f1 (ppm)

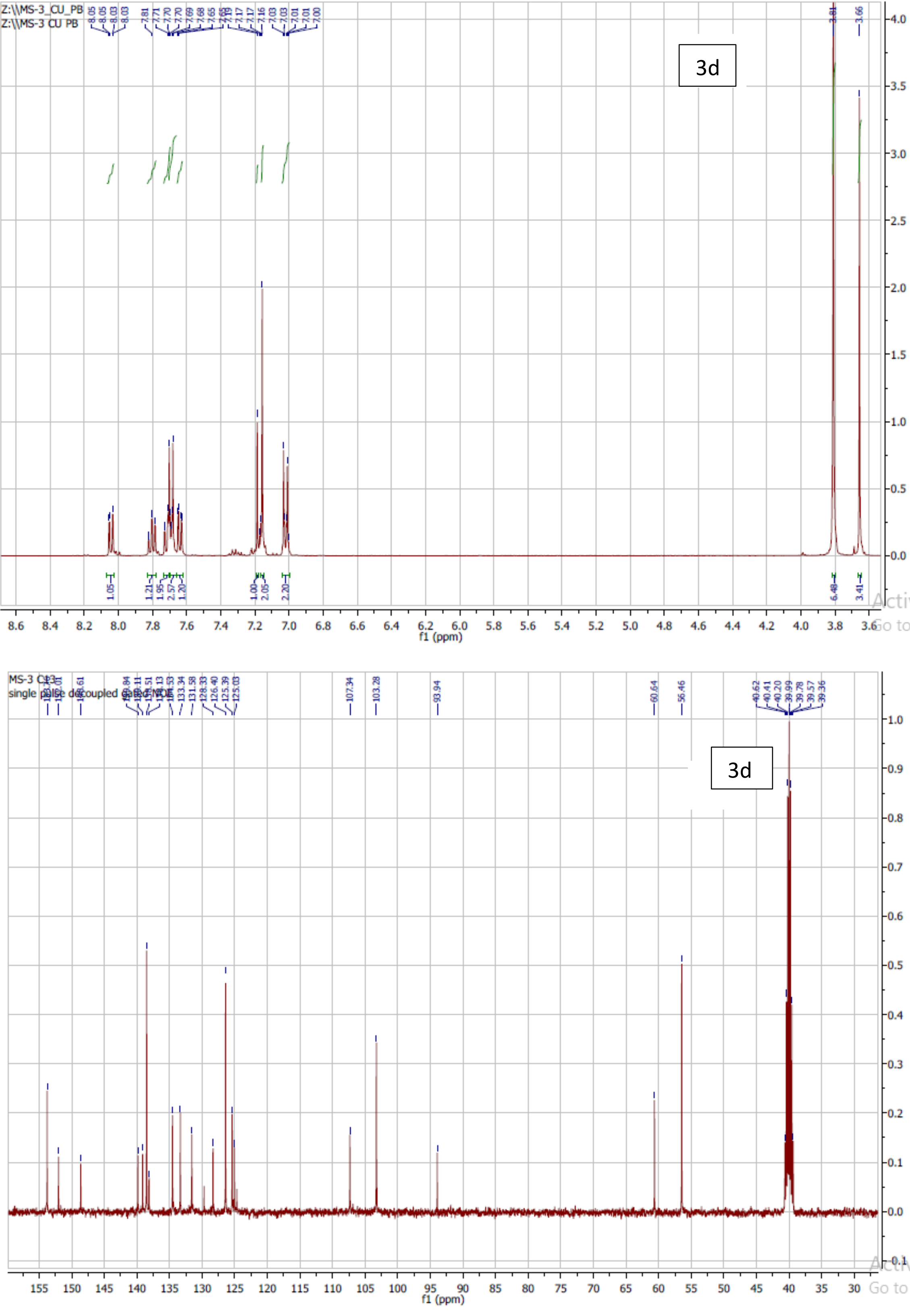

2d

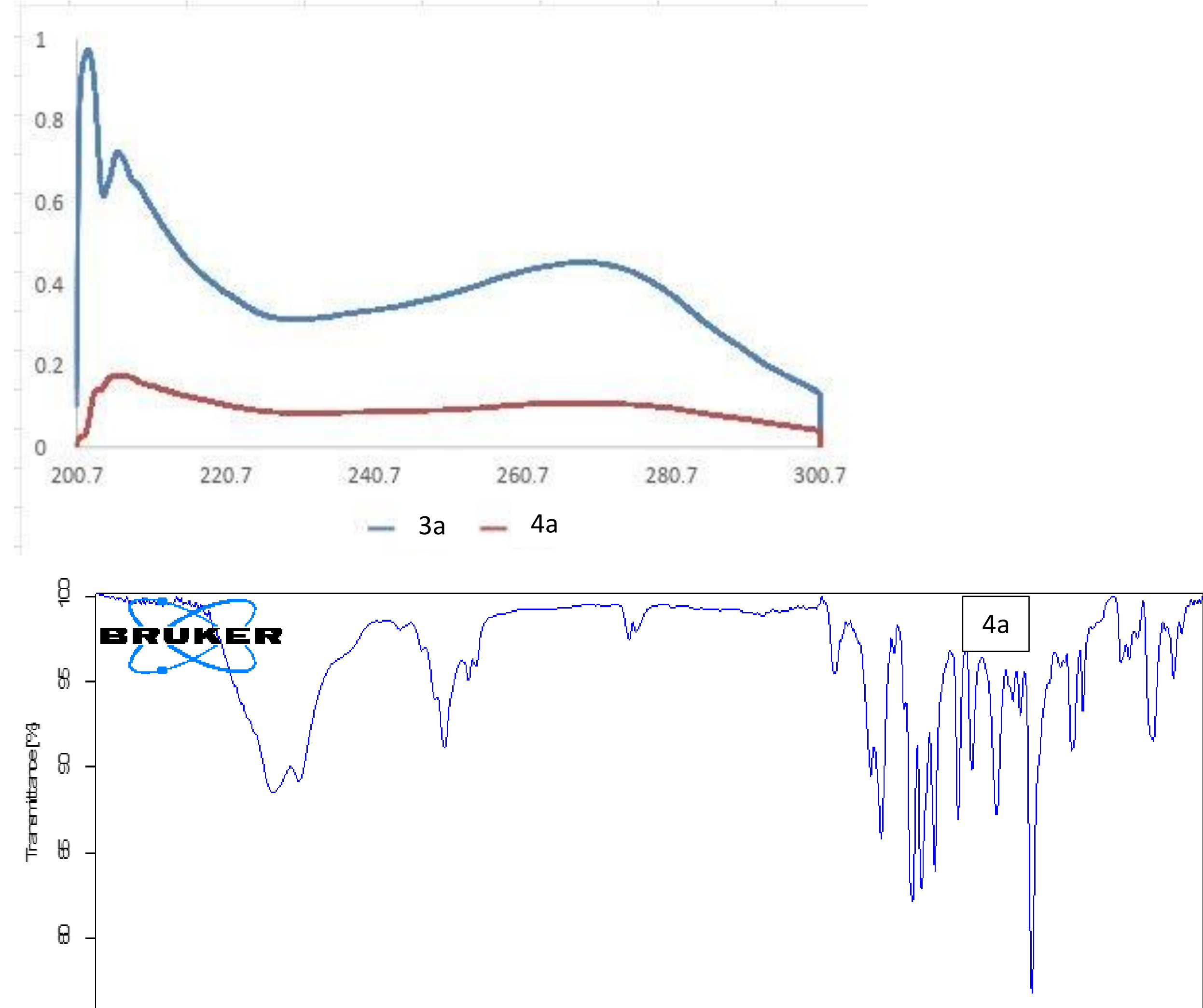

153.41
147.97
147.68
144.01
138.85
137.09
134.88
129.39
129.32
128.00
125.84
119.21
113.03
103.58
79.67
79.34
79.01
60.53
60.42
56.32
43.12
40.67
40.46
40.25
40.05
39.84
39.63
39.42
2d
200
180
160
140
120
100
80
60
40
20
0
ppm

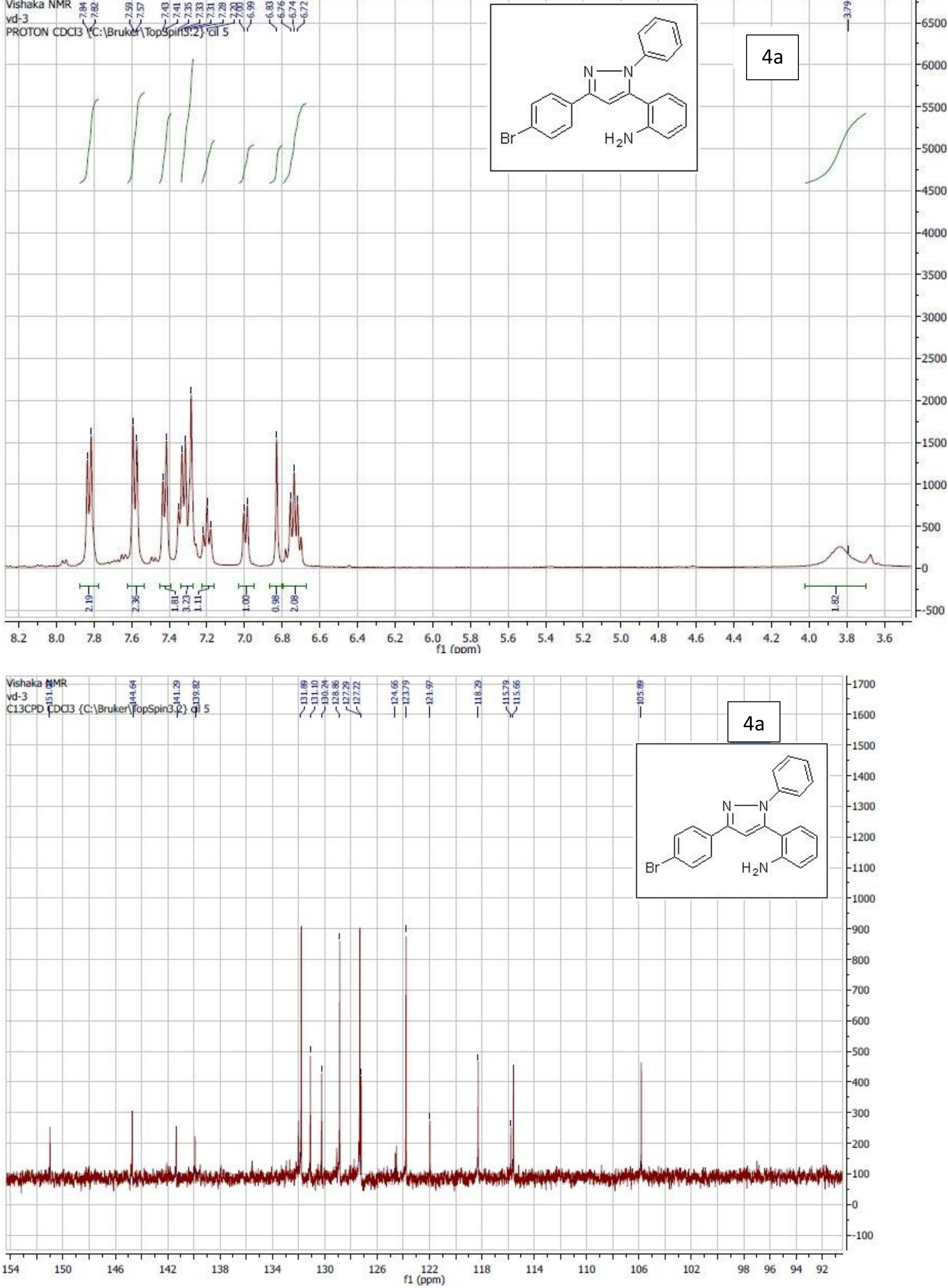

May21-2018
DMS-VR-2
8.17
8.15
7.72
7.70
7.56
7.54
7.52
7.48
7.46
7.21
6.96
6.94
6.83
6.82
6.81
4.15
4.13
4.12
4.09
3.87
3.19
3.17
3.15
3.14
2e
0.94
2.00
3.00
2.06
4.10
1.00
1.01
1.03
3.05
1.00
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
f1 (ppm)

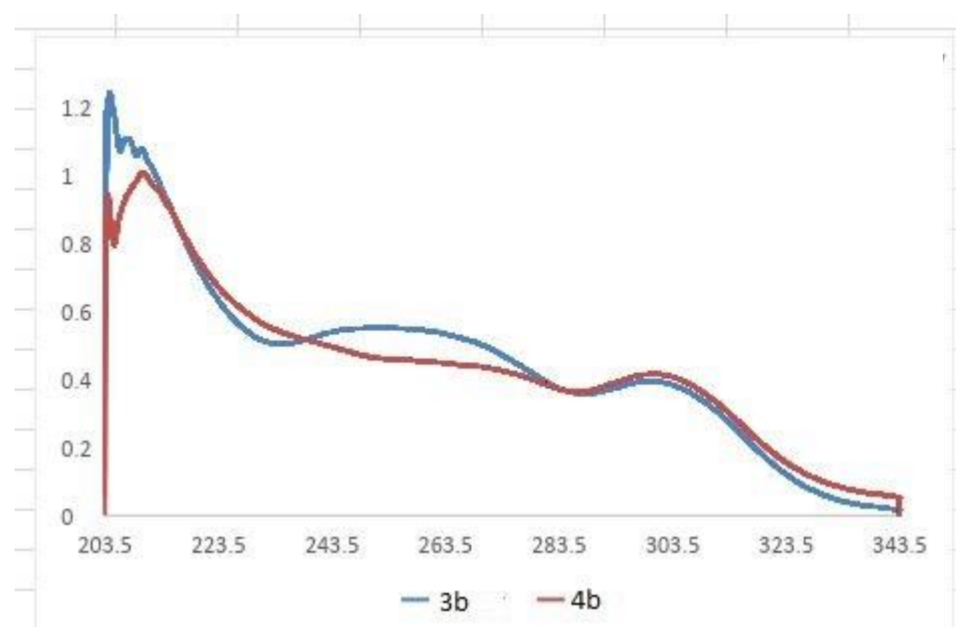

May21-2018
DMS-VR-2
160.40
147.49
147.22
144.27
137.89
134.41
129.04
128.51
128.35
127.36
125.43
125.14
119.11
114.09
112.82
60.82
55.44
43.30
2e
f1 (ppm)


BRUKER
3d
Transmittance [%]
Wavenumber cm-1

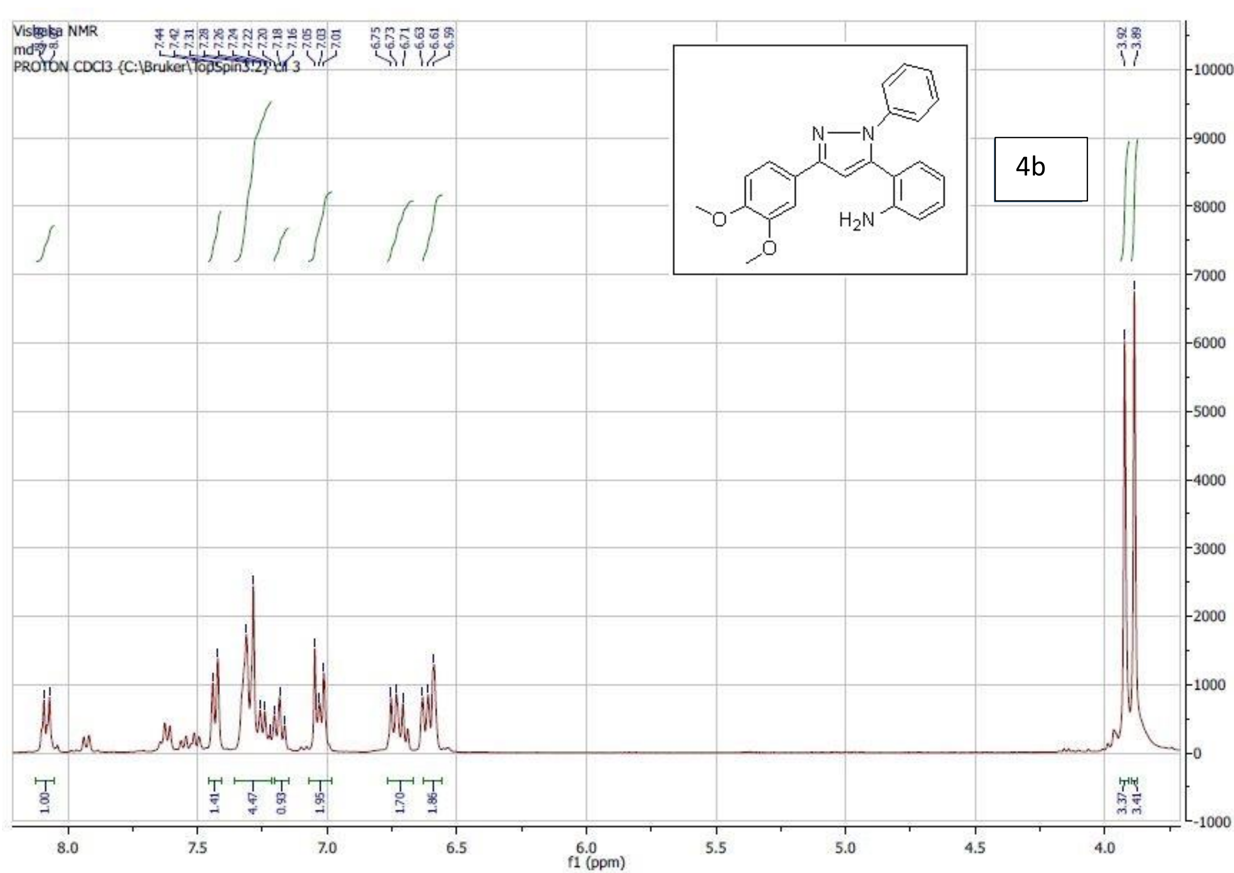

3d
f1 (ppm)
3d
f1 (ppm)


1
0.8
0.6
0.4
0.2
0
200.7
220.7
240.7
260.7
280.7
300.7
3a
4a
BRUKER
4a
Transmittance [%]
Wavenumber cm-1
3500
3000
2500
2000
1500
1000

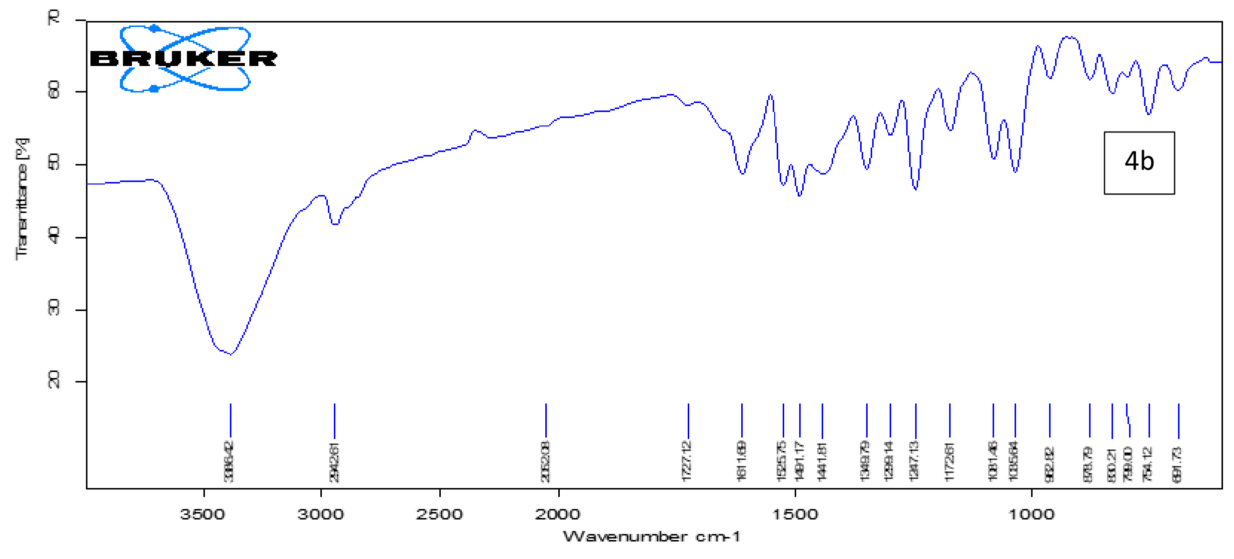

Vishaka NMR
vd-3
PROTON CDCl3 {C:\Bruker\TopSpin3.2} cil 5
4a
Br
H2N
N
N
f1 (ppm)
Vishaka NMR
vd-3
C13CPD CDCl3 {C:\Bruker\TopSpin3.2} cil 5
4a
f1 (ppm)

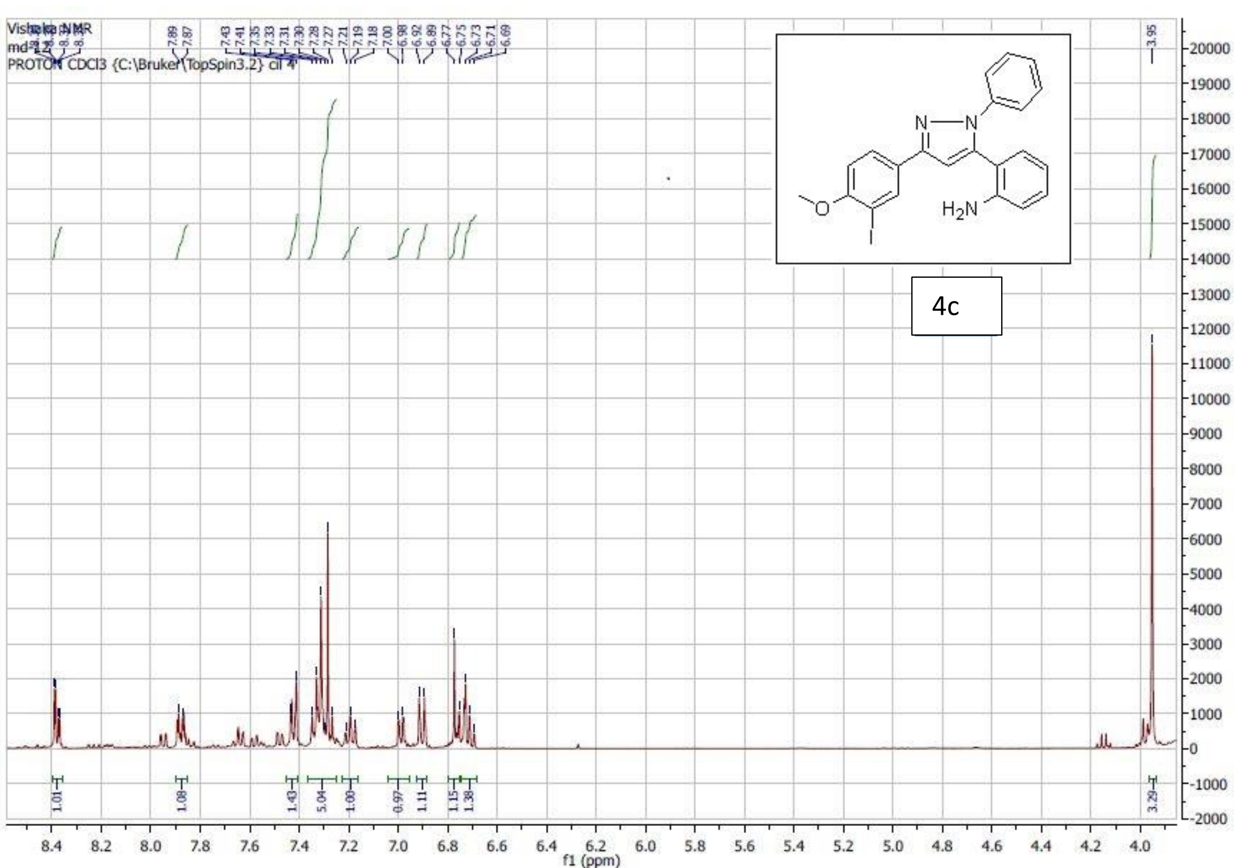


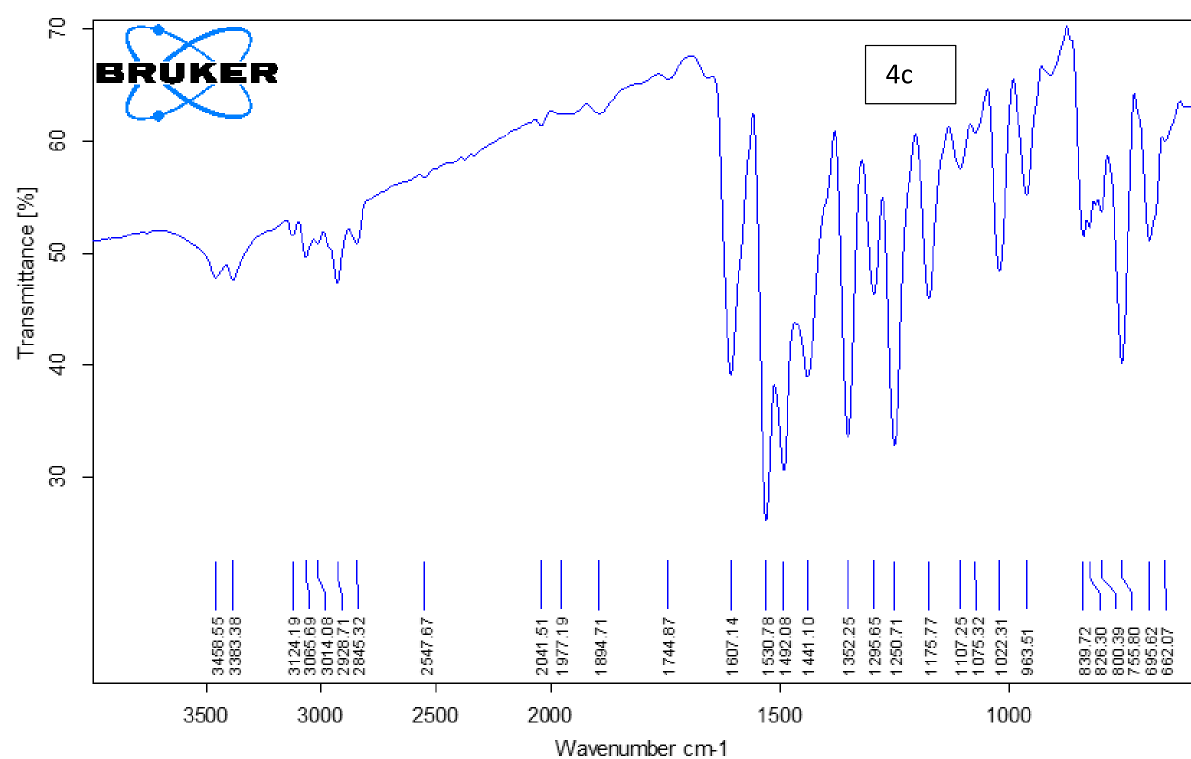
1.2
1
0.8
0.6
0.4
0.2
0
203.5
223.5
243.5
263.5
283.5
303.5
323.5
343.5
3b
4b



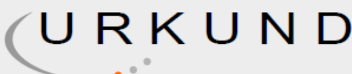
7.44
7.42
7.31
7.28
7.26
7.24
7.22
7.20
7.18
7.16
7.05
7.03
7.01
6.75
6.73
6.71
6.63
6.61
6.59
3.92
3.89
N
N
H2N
4b
1.00
1.41
4.47
0.93
1.95
1.70
1.86
3.37
3.41
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
f1 (ppm)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000


BRUKER
Transmittance [%]
Wavenumber cm-1
4b


PROTON CDCl3 {C:\Bruker\TopSpin3.2}
N
N
O
I
H2N
4c
f1 (ppm)



BRUKER
4c
Transmittance [%]
70
60
50
40
30
3458.55
3383.38
3124.19
3065.69
3014.08
2928.71
2845.32
2547.67
2041.51
1977.19
1894.71
1744.87
1607.14
1530.78
1492.08
1441.10
1352.25
1295.65
1250.71
1175.77
1107.25
1075.32
1022.31
963.51
839.72
826.30
800.39
755.80
695.62
662.07
3500
3000
2500
2000
1500
1000
Wavenumber cm-1



Urkund Analysis Result

Analysed Document: vishakha_thesis.docx (D39121141)

Submitted: 5/18/2018 1:38:00 PM

Submitted By: nonakalra@gmail.com

Significance: 6 %

Sources included in the report:

https://www.cancerwa.asn.au/resources/2016-08-10-understanding-chemotherapy.pdf
http://shodhganga.inflibnet.ac.in/bitstream/10603/62280/5/chapter%203.pdf
http://www.ijcrm.com/publish_article/edition_3/I0134145.pdf

Instances where selected sources appear:

8