MOLECULAR MODELLING, SYNTHESIS AND EVALUATION OF PYRIDAZINE DERIVATIVES AS POTENT ANTI-CANCER AGENTS

Dissertation Submitted to

The Tamil Nadu Dr. M. G. R. Medical university, Chennai,

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MASTER OF PHARMACY
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Submitted by

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CERTIFICATE

This is to certify that the dissertation work entitled **"Molecular Modelling, Synthesis and Evaluation of Pyridazine derivatives as Potent Anti Cancer agents"** submitted by **Register Number: 261425813** is a bonafide work carried out to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, in partial fulfillment for the Degree of **MASTER of PHARMACY** at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, during the academic year **2015-2016**.

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DECLARATION

I do hereby declare that the dissertation work entitled **"Molecular Modelling, Synthesis and Evaluation of Pyridazine derivatives as Potent Anti Cancer agents"** submitted to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy (Pharmacology),** was carried out at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, during the academic year **2015-2016**.

Place:

Date : **Register Number: 261425813**

EVALUATION CERTIFICATE

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Examination Centre: KMCH College of Pharmacy, Coimbatore.
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ABBREVIATIONS

ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
ALP	Alkaline phosphatase
BSA	Bovine serum albumin
CAT	Catalase
CADD	Computer Aided Drug Design
COX-2	Cyclooxygenase-2
DMH	1,2-Dimethyl hydrazine
DNA	Deoxyribonucleic acid
DTNB	5,5- Dithiobis (2-Nitrobenzoic acid)
XP	Extra-precision
EDTA	Ethylene diamine tetra acetic acid
GLIDE	Grid-based Ligand Docking with Energetics
GPx	Glutathione peroxidase
GSH	Glutathione reduced
g	Gram
FBS	Foetal bovine serum
Hb	Haemoglobin
H_2O_2	Hydrogen peroxide
IDC	Invasive (or Infiltrating) ductal carcinoma
ILC	Invasive (or Infiltrating) lobular carcinoma
IBC	Inflammatory breast cancer
IAEC	Institution of Animal Ethical Committee
i.p	Intra peritoneal
LCIS	Lobular carcinoma in situ
LPO	Lipid peroxidation

L	Litre
MTT	3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide
mL	Millilitre
mg	Milligram
Na CMC	Sodium carboxy methyl cellulose
p.o	Per oral
PBS	Phosphate buffered saline
PDB	Protein Data Bank
RBC	Red blood cells
SD	Standard Deviation
SEM	Standard Error Mean
SGOT	Serum glutamate oxalo acetate transaminase
SGPT	Serum glutamate pyruvate transaminase
SOD	Superoxide dismutase
SP	Standard Precision
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TLC	Thin Layer Chromatography
TRIS	Tris (hydroxymethyl) amino methane
U/L	Units per litre
U/ml	Units per millilitre
VEGF	Vascular Endothelial Growth Factor
µL	Microlitre
µmol	Micromole
WBC	White blood cells

ABSTRACT

The present study was designed to synthesize and investigate the cytotoxic and therapeutic effects of Pyrazolo benzpyridazine derivatives against DMH induced colon carcinoma in Sprague Dawley(SD) Rats. Docking studies were performed with VEGFR2 in order to understand the interaction of ligand with targeted receptor. Based on docking scores and chemical availability, 6 compounds were synthesised and confirmed through spectral analytical techniques (UV, IR, NMR and MASS). DPPH and ABTS antioxidant activity were performed in synthesised compounds and they showed good antioxidant activity. They were screened against Human Colon Cancer (HT29) cell line. Out of these, Pz-5 was found to be potent with promising IC_{50} Value of 27.64 µM against HT29 cell line. From the acute toxicity studies conducted on Female Wistar rats, the compound PZ-5 showed to be safe upto the dose of 2000mg/kg. Further *in vivo* studies was carried out for PZ-5 by inducing the colon carcinoma with the chemical carcinogen DMH(20mg/kg) for 4months in SD Rats and therapeutic effect of the compound PZ-5 on various haematological and enzymatic parameters were studied by treating with two dose levels (100mg/kg,200mg/kg) for 30days. The compound PZ-5 moderately increased the *in vivo* antioxidant levels like Reduced Glutathione **(**GSH), Glutathione peroxidise (GPx), Catalase (CAT), Super oxide dismutase **(**SOD), and Total protein, and reduced the lipid peroxidation in a dose dependent manner. The blood cell count, serum ALP, TCA cycle enzyme succinate dehydrogenase levels were also moderately altered on treatment with the compound at a dose of 200mg/kg as compared with negative control group in a dose dependent manner. These results suggested that the compound has a significant cytotoxic activity against colon cancer.

1. INTRODUCTION

Among the entire diseases, cancer ranks high as a major killer worldwide.[1] Cancers are a large family of diseases that includes the abnormal cell growth with the potential to invade or spread to other parts of the body[2]**.** The term cancer derives from the Greek word (Karkinoma) for crab, by Hippocrates used to describe the appendage-like projections extending from tumours. Cancer refers to a disease of cells that show unlimited proliferation, dedifferentiation, invasiveness and the ability to metastatis .The branch of science dealing with the study of tumours or neoplasms is known as oncology. [3]

EPIDEMIOLOGY OF CANCER [3]

In 2012 about 14.1 million of new cancer cases occurred globally. It accounts about 8.2 million deaths or 14.6% among all human deaths. The most common category of cancer in males are prostate cancer, colorectal cancer, lung cancer, and stomach cancer, and in females, the most common types are lung cancer ,breast cancer, colorectal cancer, and cervical cancer. In children, brain tumors and acute lymphoblastic leukaemia are most common except in Africa where non-Hodgkin lymphoma appears more often. In 2012, about 165,000 children under the age of 15 years were diagnosed with cancer. The threat of cancer increases appreciably with age and may occur generally in developed countries. [3]

THE SIX HALL MARKS OF CANCER[3]

1. Cell growth and multiplication without the proper signals
2. Continuous cell division and growth even when there are signals bring to an end
3. Avoidance of the programmed cell death
4. Unlimited number of cell divisions
5. Promoting blood vessel construction
6. Invasion of tissue and development of metastases[3]

TYPES OF TUMOURS [4]

Tumors may be benign or malignant. Benign tumours are generally localized, slow growing, resemble normal cells, and are usually not harmful. The terms cancer or malignant neoplasm or malignant tumour are synonymous. They proliferate rapidly, manifest dedifferentiation, invasiveness, and the capacity to metastasis. They cause damage on the surrounding cells and are harmful if left untreated.

Cancers are classified based on

- The Origin of the tumor.

These types include:

- ❖ Benign tumours names usually end with "oma", for example Papilloma (from surface epithelium), Adenoma (from glandular epithelium), Melanoma (from pigment cells), Myoma (from muscle tissue), Fibroma (from fibrous tissue) Neurofibroma (from nerve sheath) and A*leiomyoma* (from smooth muscle cells) ,etc.
- ❖ Malignant neoplasms are either Solid tumours or Haematological malignancies.

- Carcinoma: Derived from epithelial cells, most common cancers, mainly in the aged, in the breast, prostate, lung, pancreas, and colon.
- Blastoma: Derived from immature "precursor" cells or embryonic tissue and are more common in children than in older adults.
- Leukemia and lymphoma: These two types of tumours arise from hematopoietic (blood-forming) cells and be likely to mature in the blood and lymph nodes, respectively. In children, leukemia is the most common type of cancer accounts for about 30%.
- Germ cell tumor: Derived from pluripotent cells, occurs in the ovary or the testicle (dysgerminoma and seminoma, respectively).
- Sarcoma: Arises from connective tissue (i.e. nerve, cartilage, bone, fat), which develops from cells originating in mesenchymal cells outer the bone marrow.

Types of cancer classified by body system

Cancer has the potential to affect each organ in the body. The cells inside the malignant tumours have the capability to attack surrounding tissues and organs, thus spreading the disease.

1. Blood cancer: These cancers are also known as leukemia or lymphoma.
    - Leukemia.
    - Waldenstrom's Macroglobulinemia
    - Lymphoma.
    - Multiple Myeloma
2. Bone cancer: It is rare type of cancer. It affects both adults and children. Common type of cancer are
    - Ewings sarcoma
    - Osteosarcoma
3. Brain cancer: Brain cancers are malignant or benign. They affect both children and adults. It spread only in brain.
    - Brain tumour
    - Brain Stem Glioma, Childhood
    - Cerebellar Astrocytoma, Childhood
    - Cerebral Astrocytoma / Malignant Glioma, Childhood
    - Ependymoma, Childhood
    - Medulloblastoma, Childhood
4. Breast cancer: It is a common type of cancer, mainly affect females. Common types of breast cancers are
    - Ductal carcinoma in situ
    - Lobular carcinoma in situ
    - Inflammatory breast cancer
    - Paget’s disease of the nipple
5. Digestive cancers: Also known as gastrointestinal cancers. It is a broad group of cancer that affects all the parts from oesophagus to anus. All cancers are specific and have its own symptoms. They are
    - Anal cancer
    - Bile Duct Cancer, Extra hepatic
    - Carcinoid Tumour, Gastrointestinal
    - Colon Cancer
    - Gall bladder Cancer
    - Oesophageal Cancer
    - Liver Cancer

- Pancreatic Cancer
- Rectal Cancer
- Small Intestine Cancer
- Stomach (Gastric) Cancer

6. Endocrine cancers: The most common type is thyroid cancer. The other types are
    - Adrenocortical Carcinoma
    - Islet Cell Carcinoma (Endocrine Pancreas)
    - Parathyroid Cancer
    - Pheochromocytoma
    - Pituitary Tumour
    - Thyroid Cancer

7. Eye Cancer: It will affect both children and adults.
    - Melanoma, Intraocular
    - Retinoblastoma

8. Genitourinary cancers: It affects the male genitalia and urinary tract.
    - Bladder Cancer
    - Kidney (Renal Cell) Cancer
    - Penile Cancer
    - Prostate Cancer
    - Renal Pelvis and Ureter Cancer, Transitional Cell
    - Testicular Cancer
    - Urethral Cancer
    - Wilms' Tumour and Other Childhood Kidney Tumours

9. Gynaecological cancers: It will affect the organs of female reproductive system.
    - Cervical Cancer
    - Endometrial Cancer
    - Ovarian Cancer
    - Vaginal Cancer
    - Vulvar Cancer

10. Head and Neck cancers: This cancer affects the moist surface of neck and head. Cigarette smoking plays important role in this cancer.

- Hypo pharyngeal Cancer
- Laryngeal Cancer
- Lip and Oral Cancer
- Metastatic Squamous Neck Cancer
- Nasopharyngeal Cancer
- Oropharyngeal Cancer
- Paranasal Sinus and Nasal Cavity Cancer
- Parathyroid Cancer
- Salivary Gland Cancer

11. Respiratory cancers: Cigarette smoking is the main reason for cancer affecting respiratory system.

- Lung Cancer
- Malignant Mesothelioma
- Thymoma and Thymic Carcinoma

12.Skin cancer: The UV rays from sun are one of the main reasons for skin cancer.

- Cutaneous T-Cell Lymphoma
- Melanoma
- Non-Melanoma Skin Cancer

SYMPTOMS OF CANCER [5]

It is important to note that some types of cancer do not present any symptoms until they are in advanced stages. Because of this reason cancer screening and its risk assessment are vital for cancer prevention and its early detection. The common symptoms are,

- **Persistent fatigue**

Fatigue is one of the most commonly experienced cancer symptoms. It is usually more common in the advanced conditions, but still occurs in the early stages of some cancers. Fatigue is a symptom of both malignant and non malignant conditions.

- **Weight loss**

It can be a red flag for many illnesses, including cancer. This type of weight loss can occur with or without loss of appetite. Weight loss can be a symptom of cancer, but is also a symptom of many other illnesses, too.

- **Pain**

Typically, pain is not an early symptom of cancer, except in some cancer types like those that are spread to the bone. Pain generally occurs when cancer spreads and begins to affect other organs and nerves. Lower back pain is a cancer symptom that is associated with ovarian cancer and colon cancer. Shoulder pain can also be a symptom of lung cancer. Pain in the form of headaches can be associated with brain tumours (malignant and benign). Stomach pains can be related to types of cancer like stomach and pancreatic cancer.

- **Fever**

Fever is a very non-specific symptom of many mild to severe conditions, including cancer. Fevers are commonly associated with types of cancers that affect the blood, like leukemia and lymphoma, but are also common in people who have metastasis cancer.

- **Bowel changes**

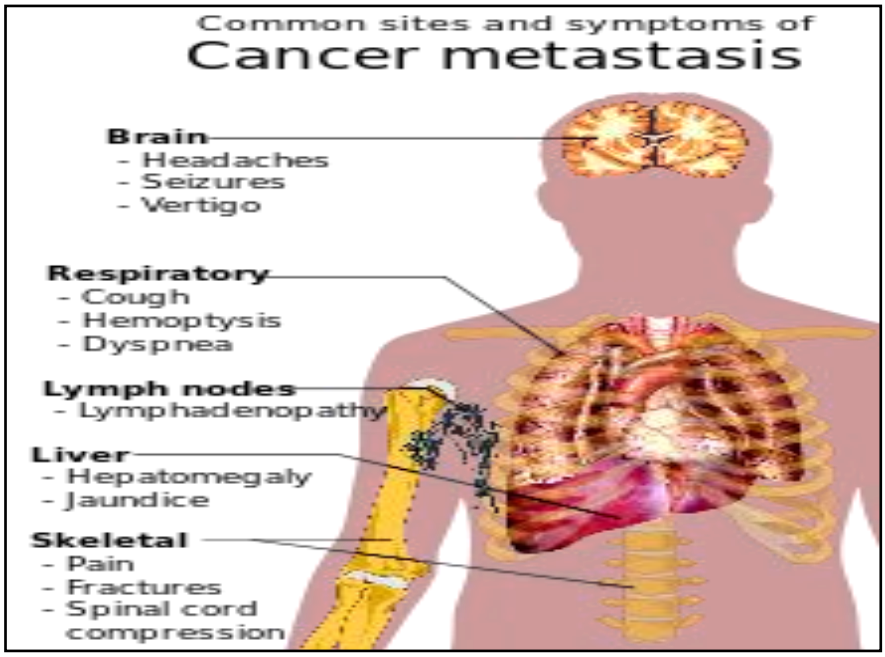
The symptoms like constipation, diarrhoea, blood in stools, gas, thinner stools, or overall changes in bowel habits are commonly associated with colon cancer, but are also related to other cancer types.

- **Chronic cough**

A persistent, new cough or that becomes worse needs to be evaluated by a doctor. A chronic cough with blood and mucus can be a symptom of lung cancer.These are vague symptoms of cancers and they can also be experienced by most people with cancer at various stages of their disease, but are also linked to many other non-cancerous conditions.

- Based on the location of the tumour the symptoms of cancer metastasis is depicted in the figure 1.[3]

Fig.1 shows the Symptoms of cancer metastasis based on location of tumour



ETIOLOGY OF CANCER [3]

Cancer is a diverse class of disease which varies widely in their causes and biology. Among all known cancers, the common thread is the attainment of abnormalities in the genetic material of the cancer cell and its progeny.

The cancer pathogenesis can be categorized into three areas of interest.

1. The agents or events which effect or assist inherited changes in cells intended to become cancer.

2. It is significant to uncover the exact nature of the inherited damage, and the genes which are affected by it.

3. As a result of these genetic changes on the cell biology, both in initiating the essential properties of a cancer cell, and in facilitating further genetic events, leads to further development of the tumor.

Chemicals

Exposure to certain substances called carcinogens for example,

1. Tobacco smoking causes 90% of lung cancer which also results cancer in the head, larynx, head, neck, bladder, stomach, kidney, esophagus and pancreas.

2. Alcohol exposure results in cancer of the liver and of the digestive tract.

3. Cancer related substance at work.

In every year, at least 200,000 people die worldwide from cancer related to their workplaces.

Diet and exercise

- About 30-35% of cancer deaths, are related to diet, physical inactivity, and obesity.
- Excess body weight and is associated with the progression of major types of cancer and is a factor in 14–20% of all cancer deaths.
- Some specific foods are linked to specific cancers. For example, gastric cancer is more common in Japan due to its high-salt diet and colon cancer is more common in the United States.

Infection

Oncovirus that can cause cancer include

- Human papillomavirus (cervical carcinoma),
- Epstein–Barr virus (B-cell lymphoproliferative disease and nasopharyngeal carcinoma),
- Kaposi's sarcoma herpes virus (Kaposi's sarcoma and primary effusion lymphomas),
- Hepatitis B and hepatitis C viruses (hepatocellular carcinoma), and
- Human T-cell leukemia virus-1 (T-cell leukemias).
- Bacterial infection may also induce cancer include *Helicobacter pylori*-induced gastric carcinoma.

Radiation

- It includes both ionizing radiation and non-ionizing ultraviolet radiation .
- Sources of ionizing radiation include medical imaging and radon gas.
- Prolonged exposure to non-ionisng ultraviolet radiation from the sun can lead to melanoma and other skin malignancies.

Heredity

- The majority of cancers are non-hereditary ("sporadic cancers").
- Hereditary cancers are mainly caused by an inherited genetic defect.
- Certain inherited mutations in the genes *BRCA1* and *BRCA2* with a more than 75% risk of breast cancer and ovarian cancer, and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome), which is present in about 3% of people with colorectal cancer, among others.

Physical agents

- Prolonged exposure to asbestos, naturally occurring mineral fibers that are a major cause of mesothelioma, which is a cancer of the serous membrane surrounding the lungs.

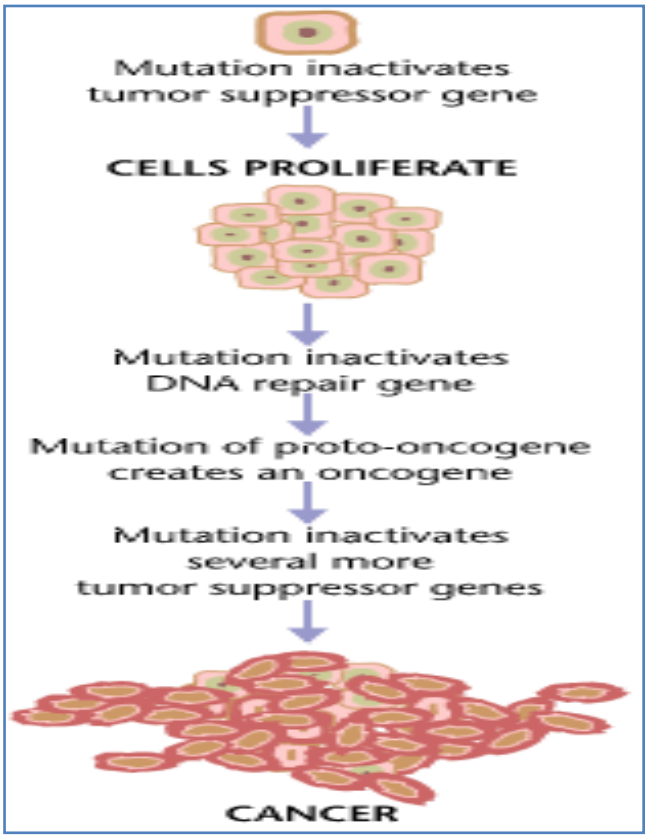
Hormone

- Insulin-like growth factors and their binding proteins play a major role in cancer cell proliferation, differentiation and apoptosis, suggesting possible involvement in carcinogenesis.
- Hormones are important agents in sex-related cancers, such as cancer of the breast, endometrium, prostate, ovary, and testis, and also of thyroid cancer and bone cancer.

PATHOPHYSIOLOGY [9]

Cancers are caused by a sequence of mutations, which causes the variation of the behavior of the cells. Normal cell mutated into cancer cell, cause the failure of regulation of cell growth and the carcinogenesis process is depicted in figure 2.

Figure 2: Carcinogenesis process



THE GENESIS OF A CANCER CELL [9]

A normal cell turns into a cancer cell because of one or more mutations in its DNA, which can be inherited or acquired. The cancer progression is a complex multistage events, involving not only more than one genetic change but usually also other,epigenetic factors (co-carcinogen and tumour promotor effects, etc.) that are indirectly produces cancer by genetic mutations.

- Two major catagories of genetic change that lead to cancer:
  - The activation of proto-oncogene to oncogenes
  - The activation of tumour suppressor genes.

    These changes are a result of point mutations, gene amplication or chromosomal translocation, often due to the action of certain viruses or chemical carcinogenes.

a. Activation of proto-oncogenes to oncogenes

Proto-oncogenes are genes that normally control cell division, apoptosis and differentiation but which can be converted to oncogenes by viral or carcinogen action.These include :

- Her2/neu(breast cancer)
- Bcr-abl (chronic myelocytic leukemia,B-cell acute lymphocytic leukemia)

Oncogenes are classified based on the functional and biochemical properties of protein products of their proto-oncogenes.

1. Growth factor
2. Growth factor receptor
3. Signal transducers
4. Transcription factor
5. Other including programmed cell death regulators

b. Inactivation of tumour supressor genes

Normal cells contain genes that have the ability to supress malignant change termed tumour suppressor genes and the mutations of these genes are involved in many different cancers. The loss of function of tumour suppressor genes can be the important event in carcinogenesis.

Eg:

RB (Retinoblastoma) gene mutations results in Retinoblastoma, Bone, Breast, Lung, Prostate and Bladder cancers.

P53 gene mutations leads to the development of Breast,Colon,Leukemia and soft tissue sarcomas.

BRCA 1 gene located in chromosome 17, mutation associated with high risk of developing Breast cancer.

About 30 tumour suppressor genes and dominant oncogenes have been identified.

THE SPECIAL CHARACTERISTICS OF CANCER CELLS [7]

➢ UNCONTROLLED PROLIFERATION

The proliferation of cancer cells is not controlled by the processes that normally regulate cell division and tissue growth. Inactivation of tumour suppressor genes or transformation of proto-oncogenes into oncogenes can confer autonomy of growth on a cell and thus result in uncontrolled proliferation by producing changes in:

- Growth factors and their receptors.
- The growth factor pathways-the cystolic and nuclear transducers.
- The cell cycle transducers –eg. Cyclins, cyclin-dependent kinases(cdks) or the cdk inhibitors
- The apoptic mechanisms that normally dispose of abnormal cells.
- Telomerase expression
- Local blood vessels, resulting from tumour-directed angiogenesis.

❖ Apoptosis and the genesis of a cancer cell

Apoptosis is programmed cell death and anti-apoptotic genetic lesions are necessary for cancer to develop. In fact development of resistance to apoptosis is a hallmark of cancer. Decreased apoptosis can be brought about by inactivation of proapototic factors or by activation of anti-apoptotic factors.

❖ Telomerase expression

Telomeres are specialized structures that cap the ends of chromosomes like the small metal tubes on the end of shoelaces protecting them from degradation, rearrangement and fusion with other chromosomes. Germline cells, stem cells and the proliferating cells of the gastrointestinal tract, bone marrow ,etc. express telomerase-an enzyme that maintains and stabilizes telomeres. Most fully differentiated somatic cells do not express telomerase, but about 95% of late-stage malignant tumours do express it and it is suggested that this enzyme can confer ‘immortality’ on a cancer cell.

❖ The control of tumour-related blood vessels

The actual growth of a solid tumour depends on the development of its own blood supply. Tumours 1-2 mm in diameter can receive nutrients by diffusion, but any

further expansion requires the development of new blood vessels angiogenesis. Angiogenesis occurs in response to growth factors produced by the growing tumours.

- **DEDIFFERENTIATION AND LOSS OF FUNCTION**

One of the main characteristics of cancer cells is that they differentiate to a varying degree in different tumours. In general, poorly differentiated cancers multiply faster and have a poor prognosis than well-differentiated cancers.

- **INVASIVENESS**

Normal cells are not found outside their 'designated' tissue of origin. For example, liver cells are not found in the bladder, and pancreatic cells are not found in the testis. This is because during differentiation and the growth of tissues and organs, normal cells develop certain spatial relationships are maintained by various tissue-specific survival factors anti-apoptotic factors. Any cells that escape accidentally lose these survival signals and undergo apoptosis.

- **METASTASIS**

Metastasis is the spread of cancer to other locations in the body by local spread, lymphatic spread to regional lymph nodes or by blood (haematogenous spread) to distant sites. The new tumors are called metastatic tumors, while the original is called the primary tumor. The symptoms of metastatic cancers depend on the location of the tumor, and can include enlarged lymph nodes (which can be felt or sometimes seen under the skin and are typically hard), enlarged liver or enlarged spleen, which can be felt in the abdomen, pain or fracture of affected bones, and neurological symptoms. Almost all cancers can metastasize. Most cancer deaths are due to cancer that has spread from its primary site to other organs (metastasized).

- **DIAGNOSIS**

Most cancers are initially identified either because of the appearance of signs or symptoms or through screening. Neither of these lead to a definitive diagnosis, which requires the examination of a tissue sample by a pathologist. These commonly include blood tests, X-rays, CT scans and endoscopy.

COLON CANCER [6]

Globally, colorectal cancer is the third most common type of cancer making up about 10% of all cases. In 2012, there were 1.4 million new cases and 694,000 deaths from the disease. It is more common in developed countries, where more than 65% of cases are found. It is less common in women than men. Colorectal cancer is the fourth most common cancer in both men and women in the United States. Most colorectal cancers grow slowly over several years, and about 1 in 20 people develop colorectal cancer.Due to early screening and improved treatments, survivorship has increased over the past 20 years.

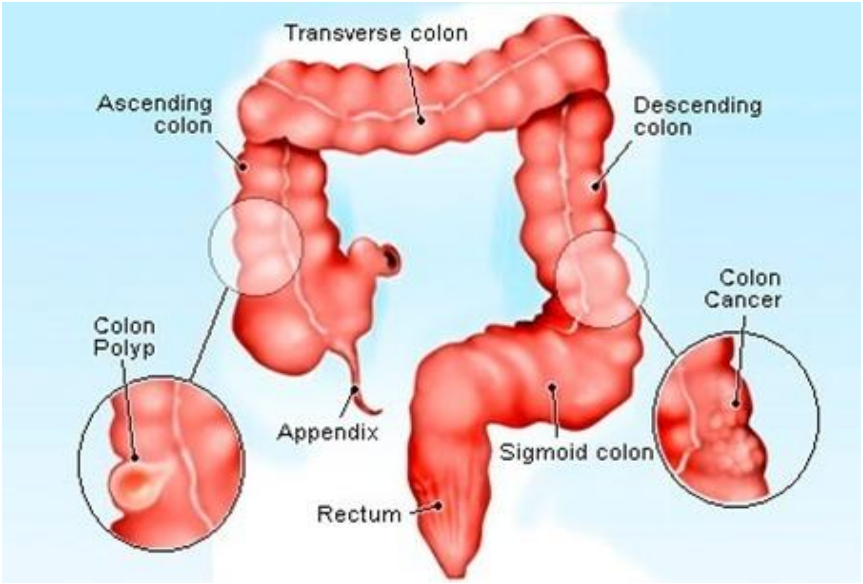
ANATOMY OF COLON [3,6]

The **large intestine**, also called the **colon** or the **large bowel**, is the last part of the digestive system in vertebrates.The ileum (last part of the small intestine) connects to the cecum (first part of the colon) in the lower right abdomen. The rest of the colon is divided into four parts and the anatomy of colon is shown in figure 3.They are:

- The ascending colon travels up the right side of the abdomen.
- The transverse colon runs across the abdomen.
- The descending colon travels down the left abdomen.
- The sigmoid colon is a short curving of the colon, just before the rectum.

The length of the adult human colon is, on average, for women 155 cm (range of 80 to 214 cm) and for men 166 cm (range of 80 to 313 cm). The average inner circumference of sections of the colon in centimeters (with ranges in parentheses) are cecum 8.7 (8.0-10.5), ascending colon 6.6 (6.0-7.0), transverse colon 5.8 (5.0-6.5), descending/sigmoid colon 6.3 (6.0-6.8) and rectum near rectal/sigmoid junction 5.7 (4.5-7.5).

Figure 3: Anatomy of colon



PHYSIOLOGY OF COLON [6]

The colon removes water, salt, and some nutrients forming stool. Muscles line the colon's walls, squeezing its contents along. Billions of bacteria coat the colon and its contents, living in a healthy balance with the body. It extracts water and salt from solid wastes before they are eliminated from the body and is the site in which flora-aided (largely bacterial) fermentation of unabsorbed material occurs. Unlike the small intestinethe colon does not play a major role in absorption of foods and nutrients. About 1.5 litres or 45 ounces of water arrives in the colon each day.[6]

COLON CANCER [6,3]

Colorectal cancer is the development of cancer in the colon or rectum (parts of the large intestine). If the cancer began in the colon, which is the first four to five feet of the large intestine, it may be referred to as colon cancer. If the cancer began in the rectum, which is the last several inches of the large intestine leading to the anus, it is called rectal cancer. Colorectal cancer starts in the inner lining of the colon and/or rectum, slowly growing through some or all of its layers. It typically starts as a growth

of tissue called a polyp. A particular type of polyp, called an adenoma, can then develop into cancer..

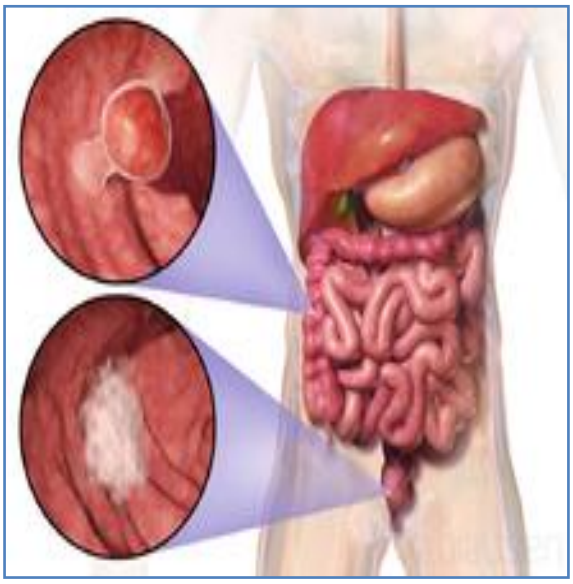
Types of colorectal cancer [6]

- Adenocarcinoma
- Gastrointestinal carcinoid tumors,
- Gastrointestinal stromal tumors,
- Primary colorectal lymphoma,
- Leiomyosarcoma,
- Melanoma and
- Squamous cell carcinoma.

SIGNS AND SYMPTOMS [3,6]

The signs and symptoms of colorectal cancer depend on the location of the tumor (Fig.No.4) in the bowel, and whether it has spread elsewhere in the body (metastasis). The classic warning signs include: worsening constipation, blood in the stool, decrease in stool caliber (thickness), loss of appetite, loss of weight and feeling tired all the time. While rectal bleeding or anemia are high-risk features in those over the age of 50, other symptoms including weight loss and change in bowel habit are typically only concerning if associated with bleeding.

Figure 4: Location of tumor



STAGING OF COLON CANCER [6]

The staging system most often used for colorectal cancer is the American Joint Committee on Cancer (AJCC) **TNM** system and is shown in figure 5 and in table 1 . The TNM system is based on

- The main (primary) **tumor** (**T**) has grown into the wall of the intestine and whether it has grown into nearby areas.
- If the cancer has spread to nearby (regional) lymph **nodes** (**N**). Lymph nodes are small bean-shaped collections of immune system cells to which cancers often spread first.
- If the cancer has spread (**metastasized**) to other organs of the body (**M**).

Figure 5: Stages of colon cancer

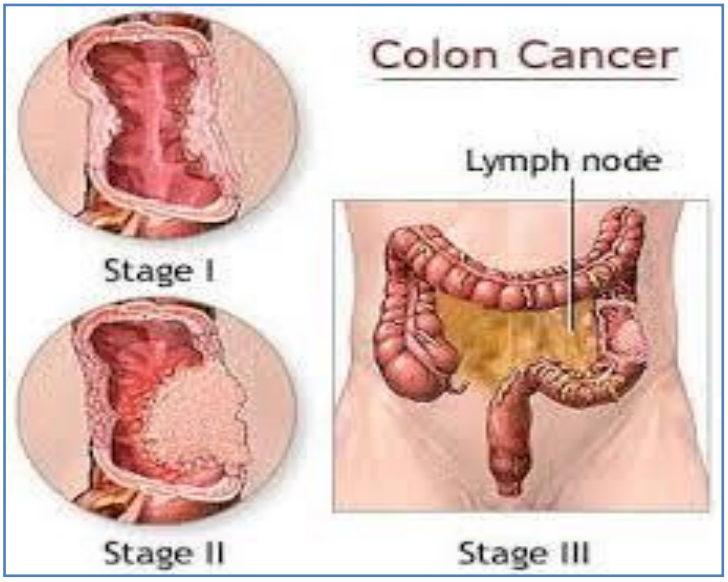


Table 1. The colon cancer staging

Stage	Stage grouping	Stage description
0	**Tis, N0, M0**	Earliest stage and also known as carcinoma in situ or intramucosal carcinoma (Tis).
I	**T1 or T2, N0, M0**	The cancer has grown through the muscularis mucosa into the submucosa (T1), and muscularis propria (T2). It has not spread to nearby lymph nodes (N0) and distant sites (M0).
IIA	**T3, N0, M0**	The cancer has grown into the outermost layers of the colon or rectum but has not gone through them (T3). It has not reached

		nearby organs like lymph nodes (N0) or to distant sites (M0).
IIB	**T4a, N0, M0**	The cancer has grown through the wall of the colon or rectum but has not grown into other nearby tissues or organs (T4a)like lymph nodes (N0) or to distant sites (M0).
IIC	**T4b, N0, M0**	The cancer is attached to or has grown into other nearby tissues or organs (T4b). It has not yet spread to nearby lymph nodes (N0) or to distant sites (M0).
IIIA	**T1 or T2, N1, M0**	It has spread to 1 to 3 nearby lymph nodes (N1a/N1b) or into areas of fat near the lymph nodes but not the nodes themselves (N1c). It has not spread to distant sites (M0).
IIIB	**T3 or T4a, N1, M0**	The cancer has grown into the outermost layers of the colon or rectum (T3) or through the visceral peritoneum (T4a) but has not reached nearby organs. It has spread to 1 to 3 nearby lymph nodes (N1a or N1b) or into areas of fat near the lymph nodes but not the nodes themselves (N1c). It has not spread to distant sites (M0).
IIIC	**T4a, N2a, M0**	The cancer has grown through the wall of the colon or rectum (including the visceral peritoneum) but has not reached nearby organs (T4a). It has spread to 4 to 6 nearby lymph nodes (N2a). It has not spread to distant sites (M0).
IVA	**Any T, Any N, M1a**	The cancer may or may not have grown through the wall of the colon or rectum (Any T). It might or might not have spread to nearby lymph nodes. (Any N). It has spread to 1 distant organ (such as the liver or lung) or distant set of lymph nodes (M1a).
IVB	**Any T, Any N, M1b**	The cancer might or might not have grown through the wall of the colon or rectum. It might or might not have spread to nearby lymph nodes. It has spread to more than 1 distant organ (such as the liver or lung) or distant set of lymph nodes, or it has spread to distant parts of the peritoneum (the lining of the abdominal cavity) (M1b).

COLORECTAL CANCER GRADES [3]

The scale used for grading colorectal cancers is from 1 to 4.

- Grade 1 (G1) means the cancer looks much like normal colorectal tissue.
- Grade 4 (G4) means the cancer looks very abnormal.
- Grades 2 and 3 (G2 and G3) fall somewhere in between

The grade is often simplified as either low grade (G1 or G2) or high grade (G3 or G4).Low-grade cancers tend to grow and spread more slowly than high-grade cancers.

ETIOLOGY AND RISK FACTORS OF COLON CANCER [3,6]

The risk factors of Colon cancer includes Diet, smoking, alcohol, lack of physical activity, family history of colon cancer and colon polyps, presence of colon polyps, exposure to radiation, and even other diseases such as diabetes and obesity.

DIET

A diet high in red, processed meat, while low in fiber increases the risk of colorectal cancer.

DISEASES

- Inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis, can increase the risk of colorectal cancer.
- Inherited genetic disorders include familial adenomatous polyposis and hereditary non-polyposis colon cancer.

GENETICS

- A number of genetic syndromes are also associated with higher rates of colorectal cancer and The most common of these is hereditary nonpolyposis colorectal cancer(HNPCC or Lynch syndrome) which is present in about 3% of people with colorectal cancer. Other syndromes include Gardner syndrome and familial adenomatous polyposis (FAP).
- A gene contribute to the potential for metastatic disease in colon cancer 1 (*MACC1*), has been associated with the proliferation, invasion and scattering of colon

cancer cells in cell culture, and tumor growth and metastasis in mice. MACC1 may be a potential target for cancer intervention.

- Epigenetic factors, such as abnormal DNA methylation of tumor suppressor promoters play a role in the development of colorectal cancer.

PATHOGENESIS OF COLON CANCER [3]

Colorectal cancer is a disease originating from the epithelial cells lining the colon or rectum of the gastrointestinal tract, most frequently as a result of mutations in the Wnt signaling pathway that increase signaling activity. The mutations can be inherited or acquired, and most probably occur in the intestinal crypt stem cell.The most commonly mutated gene in all colorectal cancer is the APC gene, which produces the APC protein that prevents the accumulation of β-catenin protein. Without APC, β-catenin accumulates to high levels and translocates (moves) into the nucleus, binds to DNA, and activates the transcription of proto-oncogenes. These genes are normally important for stem cell renewal and differentiation, but when improperly expressed at high levels, they can cause cancer. While APC is mutated in most colon cancers, some cancers have increased β-catenin because of mutations in β-catenin (CTNNB1) that block its own breakdown, or have mutations in other genes with function similar to APC such as AXIN1, AXIN2, TCF7L2, or NKD1. Further than the defects in the Wnt signaling pathway, p53 protein, produced by the *TP53* gene, normally monitors cell division and kills cells if they have Wnt pathway defects. Eventually, a cell line acquires a mutation in the *TP53* gene and transforms the tissue from an benign epithelial tumorinto an invasive epithelial cell cancer.

Other proteins deactivated in colorectal cancers are TGF-β and DCC (Deleted in Colorectal Cancer). TGF-β has a deactivating mutation in at least half of colorectal cancers. Sometimes TGF-β is not deactivated, but a downstream protein named SMAD is deactivated. DCC commonly has a deleted segment of a chromosome in colorectal cancer.

Some oncogenes are overexpressed in colorectal cancer. For example, genes encoding the proteins KRAS, RAF, and PI3K, which normally stimulate the cell to divide in response to growth factors, can acquire mutations that result in over-activation of cell proliferation. The chronological order of mutations is sometimes important. If a

previous APC mutation occurred, a primary KRAS mutation often progresses to cancer rather than a self-limiting hyperplastic or borderline lesion. PTEN, a tumor suppressor, normally inhibits PI3K, but can sometimes become mutated and deactivated.[31]

Genome-scale analysis has discovered that colorectal carcinomas can be categorized into hypermutated and non-hypermutated tumor types. In addition to the oncogenic and inactivating mutations described for the genes above, non-hypermutated samples also contain mutated CTNNB1, FAM123B, SOX9, ATM, and ARID1A. Progressing through a distinct set of genetic events, hypermutated tumors display mutated forms of ACVR2A, TGFBR2, MSH3, MSH6, SLC9A9, TCF7L2, and BRAF. The common theme among these genes, across both tumor types, is their involvement in WNT and TGF-β signaling pathways, which results in increased activity of MYC, a central player in colorectal cancer.

TARGETS OF CANCER RESEARCH [3]

Many different targeted therapies have been approved for use in cancer treatment. These therapies include hormone therapies, signal transduction inhibitors, gene expression modulators, apoptosis inducers, angiogenesis inhibitors, immunotherapies, and toxin delivery molecules.

- **Hormone therapies** slow or stop the growth of hormone-sensitive tumors, which require certain hormones to grow. Hormone therapies act by preventing the body from producing the hormones or by interfering with the action of the hormones. Hormone therapies have been approved for both breast cancer and prostate cancer.
- **Signal transduction inhibitors** block the activities of molecules that participate insignal transduction, the process by which a cell responds to signals from its environment. During this process, once a cell has received a specific signal, the signal is relayed within the cell through a series of biochemical reactions that ultimately produce the appropriate response(s). In some cancers, the malignant cells are stimulated to divide continuously without being prompted to do so by external growth factors. Signal transduction inhibitors interfere with this inappropriate signaling.
- **Gene expression modulators** modify the function of proteins that play a role in controlling gene expression.

- **Apoptosis inducers** cause cancer cells to undergo a process of controlled cell death called apoptosis. Apoptosis is one method the body uses to get rid of unneeded or abnormal cells, but cancer cells have strategies to avoid apoptosis. Apoptosis inducers can get around these strategies to cause the death of cancer cells.
- **Angiogenesis inhibitors** block the growth of new blood vessels to tumors (a process called tumor angiogenesis). A blood supply is necessary for tumors to grow beyond a certain size because blood provides the oxygen and nutrients that tumors need for continued growth. Treatments that interfere with angiogenesis may block tumor growth. Some targeted therapies that inhibit angiogenesis interfere with the action ofvascular endothelial growth factor (VEGF), a substance that stimulates new blood vessel formation. Other angiogenesis inhibitors target other molecules that stimulate new blood vessel growth.
- **Immunotherapies** trigger the immune system to destroy cancer cells. Some immunotherapies are monoclonal antibodies that recognize specific molecules on the surface of cancer cells. Binding of the monoclonal antibody to the target molecule results in the immune destruction of cells that express that target molecule. Other monoclonal antibodies bind to certain immune cells to help these cells better kill cancer cells.
- **Monoclonal antibodies that deliver toxic molecules** can cause the death of cancer cells specifically. Once the antibody has bound to its target cell, the toxic molecule that is linked to the antibody—such as a radioactive substance or a poisonous chemical—is taken up by the cell, ultimately killing that cell. The toxin will not affect cells that lack the target for the antibody i.e., the vast majority of cells in the body.

PROTEINS

- Nucleic acids and their precursors
- Tubulin (Micro tubular protein)

ENZYMES

- DNA topoisomerase-I &II
- 5- ∝ Reductase
- DNA polymerase

- Ribonucleoside diphosphate reductase
- Histone deacetylase(HDAC)
- Thymidilate synthase
- Thiomidilate synthatase

HORMONES

- Estrogens
- Testosterone
- Androgen
- Progestin

GENES

- p53
- Epidermal Growth Factor Receptor (EGFR)
- Vascular Growth Factor Receptor (VEGFR)
- Oncogene

DRUGS USED TO TREAT COLORECTAL CANCER [6]

- 5-Fluorouracil (5-FU)
- Capecitabine (Xeloda)
- Irinotecan (Camptosar)
- Oxaliplatin (Eloxatin)
- Trifluridine and tipiracil (Lonsurf)
- Cetuximab(erbitux)
- Panitumumab (vectibix®)
- Bevacizumab (avastin®)
- Ziv-aflibercept (zaltrap®)
- Regorafenib (stivarga®)
- Ramucirumab (cyramza®)

Often, 2 or more of these drugs are combined to try to make them more effective. Sometimes, chemo drugs are given along with a targeted therapy drug.

LIMITATION OF CONVENTIONAL THERAPY OF COLORECTAL CANCER [3]

Conventional treatment involves varied combinations of surgery, radiation therapy and chemotherapy. Radiation and chemotherapy are used if the cancer is untreatable or metastasized, and also as follow-up to surgery. In addition, immunotherapy and hormone therapy have been used in the treatment of certain forms of melanoma, breast and prostate cancers. Chemotherapeutic agents are cytotoxic drugs, which affect any cells in the body that are actively dividing. The side-effects can include nausea, vomiting, immunosuppression, mucositis, hepatotoxicity, nephrotoxicity, memory loss, anemia and even death. Several chemotherapeutic agents also have long-term side-effects in major organs such as the heart, lungs, kidneys and central nervous system.

To avoid these adverse effects, conventional chemotherapeutic agents must be administered at a safe, but suboptimal dose, which is insufficient to treat the cancer satisfactorily in one session. Physicians have attempted to treat cancer using these lower doses over a longer period of time, however, this is often ineffective due to the development of drug resistance by the cancerous cells. Currently, patients are given combination chemotherapy - multiple drugs at various doses to try to circumvent these problems. Newer treatments such as tumour vaccines and small-molecule therapies avoid many of the side-effects by targeting surface or intracellular proteins specific to the cancer being treated. However, these methods can only deal with very specific diseases. One such method is targeting **Angiogenesis Inhibitors**.

ANGIOGENESIS [10]

Angiogenesis is the process of generating new capillary blood vessels. Unregulated angiogenesis may cause different pathologies, such as tumor growth and metastasis . A growing tumor needs capillaries to provide nutrients and oxygen and is represented in figure 6 and 7. Vascular endothelial growth factor (VEGF), a major mediator of vascular permeability and angiogenesis, potentiates microvascular hyperpermeability, which can precede and accompany angiogenesis.

Figure 6: shows angiogenesis process in a growing tumour cell.

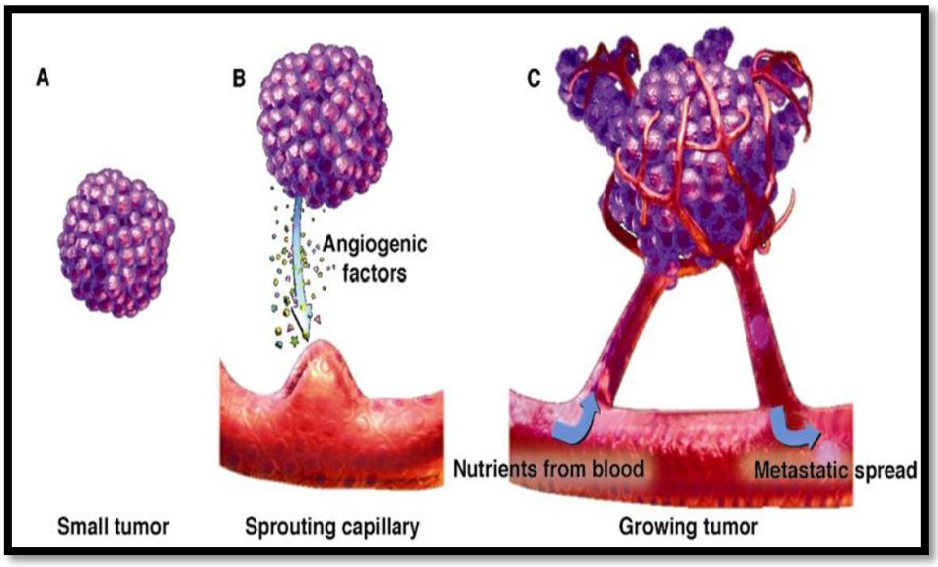
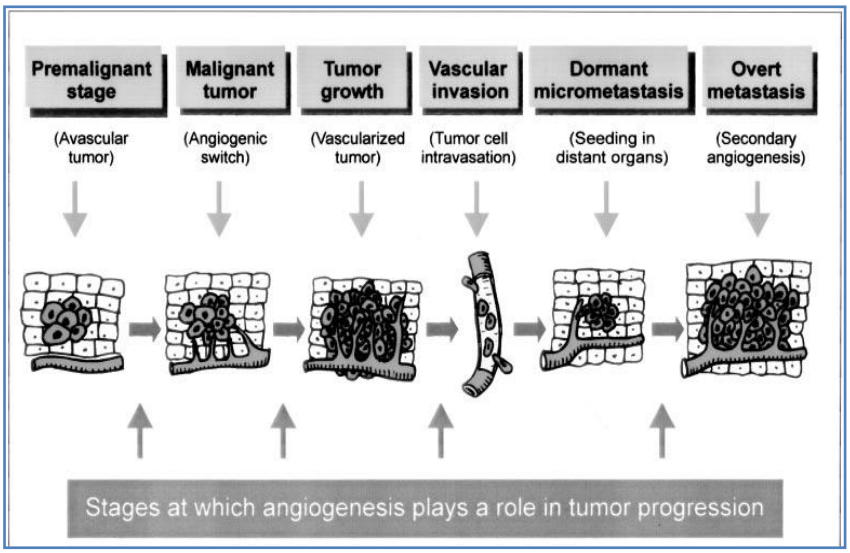


Figure 7: shows stages at which angiogenesis plays a role in tumor progression



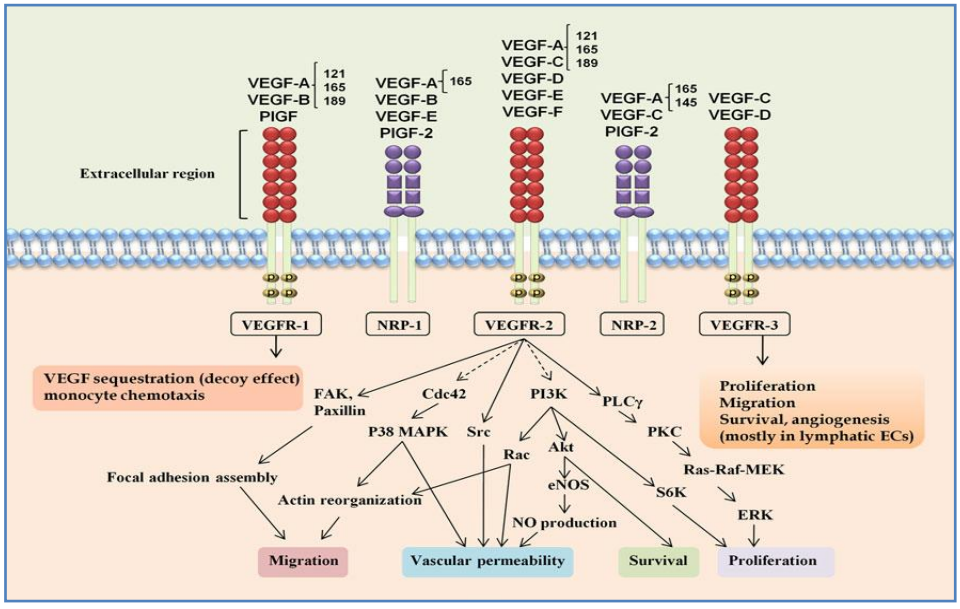
The growth factors, angiogenic enzymes, endothelial specific receptors and the adhesion molecules which are involved in the expansion of vasa vasorum are all potential therapeutic targets. Amongst growth factors Vascular Endothelial Growth Factor (VEGF) is the major pro-angiogenesis factor, which is known to stimulate various steps of endothelial angiogenic activity, such as proliferation, migration, differentiation into vessel-like tubes. Its identification and prominent position in the angiogenic process has converted VEGF to an important therapeutic target. Fibroblast growth factors(FGFs) are heparin binding proteins that are also involved in

the pathogenesis and subsequent progression of various cancer types such as endometrial cancer.

KEY REGULATORS OF ANGIOGENESIS [11,12]

VEGF is the prototypical proangiogenic molecule, a 45-kDa heparin-binding homodimeric glycoprotein and it has been implicated in several steps throughout the angiogenesis process. The VEGF family of molecules currently consists of six growth factors, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor, and three receptors, including VEGF receptor (VEGFR)-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4) and figure 8 shows the Vascular endothelial growth factor (VEGF) family and their receptors. VEGF-A is currently the most well-characterized member of the VEGF family and is composed of at least six isoforms due to alternative gene splicing . One of the most striking characteristics of VEGF is its ability to induce vascular permeability . This enhanced permeability leads to subsequent fibrin deposition in the extracellular matrix that can then serve as a scaffold for migrating endothelial cells. The three VEGFRs are transmembrane tyrosine kinases that are predominantly found on endothelial cells. The activation of VEGFR-2 by its ligands results in enhanced permeability of the vasculature and increased migration and proliferation of endothelial cells, making it also a major target for therapy.

Figure 8: **Vascular endothelial growth factor (VEGF) family and their receptors**



VEGF family members bind to specific receptor tyrosine kinases VEGFR-1, VEGFR-2, and VEGFR-3 and, through activating different cascades, exert their various biologic effects.VEGFR-2 represents the major mediator of VEGF-driven responses in endothelial cells responsible for most VEGF angiogenic activities. Neuropilins (NRP-1 and NRP-2) are known as co-receptors for VEGF.

Other Proangiogenic Factors

The platelet-derived growth factor (PDGF) family of molecules is structurally related to the VEGF family and has significant angiogenic properties invitro and in vivo.

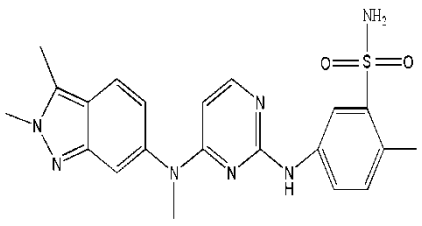
DRUGS THAT TARGET VEGF RECEPTORS [6]

Vascular endothelial growth factor (VEGF) is a protein that helps tumors form new blood vessels to get nutrients. Drugs that stop VEGF from working can be used to treat some colon or rectal cancers. These include:

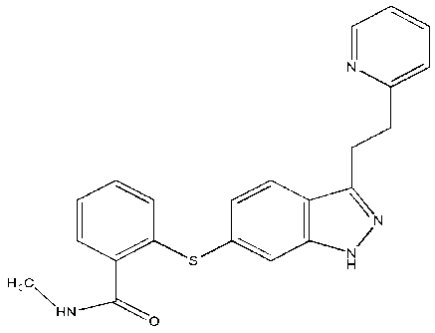
- **Bevacizumab (Avastin)**
- **Ramucirumab (Cyramza)**
- **Ziv-aflibercept (Zaltrap)**

These drugs are given as infusions into your vein (IV) every 2 or 3 weeks, typically along with chemotherapy. When combined with chemo, these drugs can often help patients with advanced colon or rectal cancers live longer.

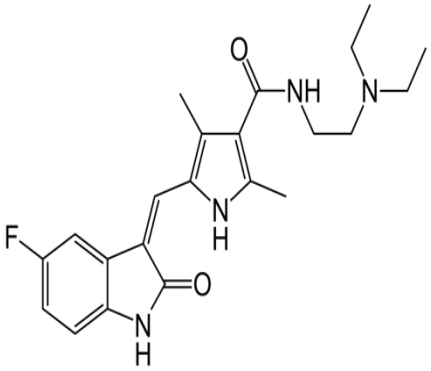
Structure of vascular endothelial growth factor receptor (VEGFR) inhibitors



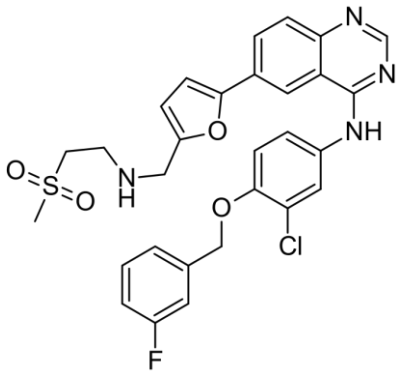
1. Pazopanib



2. Axitinib



3. Sunitib



4. Lapatinib(Tykerb)

POSSIBLE SIDE EFFECTS OF DRUGS THAT TARGET VEGF[6]

- Common side effects

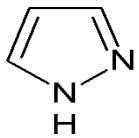
High blood pressure, tiredness, bleeding, low white blood cell counts(with increased risk of infections), headaches, mouth sores, loss of appetite, and diarrhea.

- Rare serious side effects

Blood clots, severe bleeding, holes forming in the colon (called *perforations*), heart problems, slow wound healing and allergic reaction during the infusion.

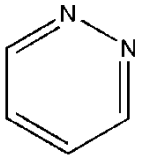
CHEMISTRY AND PHARMACOLOGICAL ACTIVITIES OF PYRAZOLE AND PYRIDAZINE

Pyrazole refers to the class of simple aromatic ring organic compounds of the heterocyclic series characterized by a 5-membered ring structure composed of three carbon atoms and two nitrogen atoms in adjacent positions**.**Being so composed and having pharmacological effects on humans, they are classified as alkaloids, although they are rare in nature. In 1959, the first natural pyrazole, 1- pyrazolyl-alanine, was isolated from seeds of watermelons.[13]A systematic investigation of this class of heterocyclic lead revealed that pyrazole containing pharmacoactive agents play important role in medicinal chemistry**.** Pyrazoles are used for their anti-inflammatory, antioxidant, ACE inhibition, antitumour, antitubercular, antiviral, antidepressant, anticonvulsant, antifungal and anti-bacterial activities.

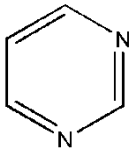


Pyrazole

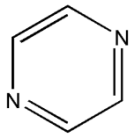
Literature survey revealed that various N-substituted pyrazoles have been implemented as antileukemic, antitumor, antiproliferative, anti-angiogenic, DNA interacting, proapoptotic, autophagy, and antitubulin agents. Moreover these compounds are capable to exert remarkable anti-cancer effects through inhibition of different types of enzymes, proteins and receptors which play critical role in cell division.[14]



Pyridazine



Pyrimidine



Pyrazine

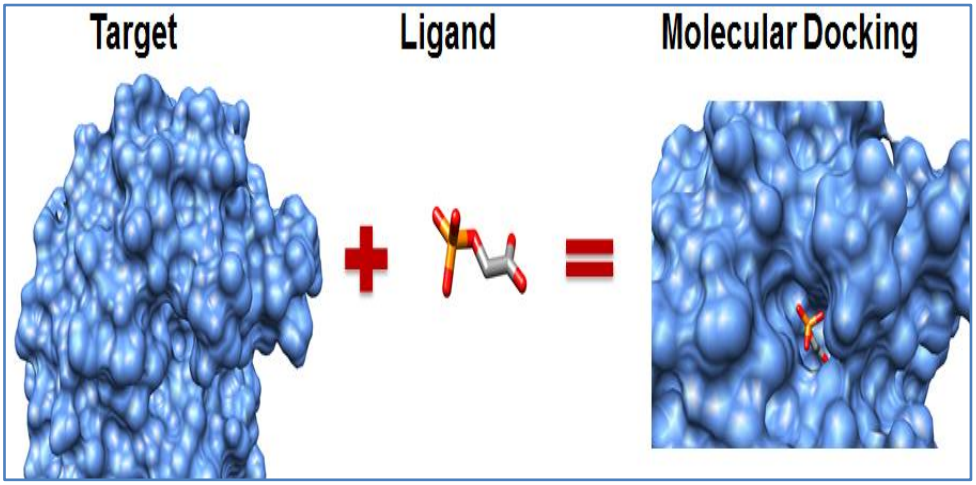
Pyridazine is a compound that belong to the class of diazines. Pyridazines are heterocyclic compounds that contain two adjacent nitrogen atoms (1,2-diazine) in the ring structure. They show a wide range of pharmacological activities and are found in a lot of natural compounds having different biological activities. There are three types of diazines: pyridazine, pyrimidine and pyrazine. The pyridazine compounds possess various pharmacological activities such as antihypertensive, antidepressant, hepatoprotective, anti-HIV, antibacterial, antimicrobial, cardiotonic, vasodilation, 5HT antagonist etc. In addition, Pyridazine derivatives has remarkable anti cancer activity against leukemia, non-small cell lung cancer, colon, central nervous system, melanoma, ovarian and breast cancer cell lines.[1]

MOLECULAR MODELLING [15]

Molecular modeling is a general term used to describe the use of computers to construct molecules and perform a variety of calculations on these molecules in order to predict the chemical characteristics and behaviour. Molecular modelling encompasses all theoretical methods and computational techniques used to model or mimic the behaviour of molecules.The benefit of molecular modelling is that it reduces the complexity of the system allowing many more particles (atoms) to be considered during simulations.

In the recent years the search of novel drugs has evolved from the process of trial and error into a sophisticated procedure including several computer based approaches. In structure based design, the structures of known target proteins are used to discover new compounds of therapeutic relevance.The approaches can be classified roughly into two categories: de novo design and docking.

Figure 9: Protein and ligand docked complex



MOLECULAR DOCKING [15]

In the field of molecular modeling, docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. Knowledge of the preferred orientation in turn may be used to predict the strength of association or binding affinity between two molecules. The association between biologically relevant molecules such as proteins, nucleic acids, carbohydrates and lipids play a central role in signal transduction. Furthermore, the relative orientation of the two interacting partners may affect the type of signal produced. Therefore, docking is useful for predicting both the strength and type of signal produced.

Docking is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets in order to predict the affinity and activity of small molecules. Hence docking plays an important role in the rational design of drugs. During the process of molecular docking the ligand binds to the particular molecule of interest and they adjust their conformations to achieve an optimised conformation to achieve an overall “best fit” and this kind of conformational adjustments resulting in the overall binding is referred to as “ induced fit”. The aim of the molecular docking

is to achieve an optimised conformation for both the protein and ligand such that the free energy of the overall system is minimised.

The application of computational methods to study the formation of intermolecular complexes is a subject of intensive research. Drug exerts its biological activity by binding to the pocket of receptor molecule (usually protein). In their binding conformations, the molecules exhibit geometric and chemical complementarily, both of which are essential for successful drug activity. Molecular docking helps in studying drug/ ligand or receptor/ protein interactions by identifying the suitable active sites in protein, obtaining the best geometry of ligand-receptor complex and calculating the energy of interaction for different ligands to design more effective ligands.

The target or receptor is either experimentally known or theoretically generated through knowledge based protein modelling or homology modelling. The molecular docking tool has been developed to obtain a preferred geometry of interaction of ligand-receptor complexes having minimum interaction energy based on different scoring functions namely only electrostatics, sum of steric and electrostatic(parameters from MMFF force field) and dock score. This utility allows one to screen a set of compounds for lead optimisation.

TOXICITY PREDICTION[16]

The investigation of ADMET properties of a compound is a crucial step in the drug development process which involves the absorption, distribution, metabolism, excretion and toxicity studies. The ADMET properties of a drug candidate have to be determined, before its proceeds into clinical trials. The usual toxicity studies were conducted in animal experiments are time-consuming and take animal lives. In silico toxicity predictions are a fast and inexpensive alternative to animal experiments. They rely on known toxicity data which is used to develop a model capable of predicting toxicities of new compounds.

PREDICTION OF RODENT ORAL TOXICITY[17]

PROTOX is a webserver for the prediction of oral toxicities of small molecules in rodents.

The prediction of compound toxicities is an important part of the drug design development process. Computational toxicity estimations are not only faster than the determination of toxic doses in animals, but can also help to reduce the amount of animal experiments. PROTOX webserver is based on chemical similarities between compounds with known toxic effects and the presence of toxic fragments.

ECOTOXICITY PREDICTION[18]

GUSAR software is used for Quantitative prediction of ecotoxicity for chemical compounds. The QSAR models were developed for the following endpoints: 96-hour fathead minnow 50% lethal concentration, 48-hour daphnia magna 50% lethal concentration, Tetrahymena pyriformis 50% growth inhibition concentration and Bioconcentration Factor.

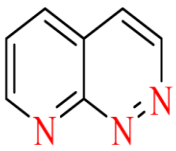
GUSAR software was developed to create QSAR/QSPR models on the basis of the appropriate training sets represented as SDfile contained data about chemical structures and endpoint in quantitative terms.

ACUTE RAT TOXICITY PREDICTION[18]

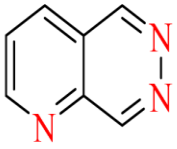
In silico prediction of LD50 values for rats with four types of administration (oral, intravenous, intraperitoneal, subcutaneous, inhalation) by GUSAR software. The training sets were created on the basis of data from SYMYX MDL Toxicity Database. They include the information about ~10000 chemical structures with data on acute rat's toxicity represented on the LD50 values.

2. REVIEW OF LITERATURE

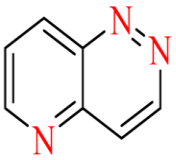
1. **Ibrahim M A *et al.*,**[19] studied the various pharmacological activities of pyridopyridazine derivatives[1] which includes anticancer, antimicrobial, analgesics,anti-histaminic,anti-asthmatics,anti-inflammatory,anti tuberculosis. These properties made them as an important scaffold for the development of new drugs.



pyrido[2,3-*c*]pyridazine

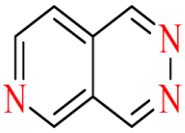


pyrido[2,3-*d*]pyridazine

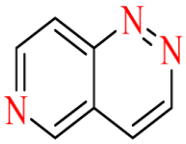


pyrido[3,2-*c*]pyridazine

pyrido[3,4-*c*]pyridazine



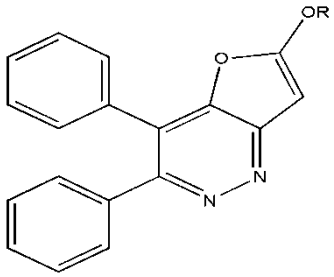
pyrido[3,4-*d*]pyridazine



pyrido[4,3-*c*]pyridazine

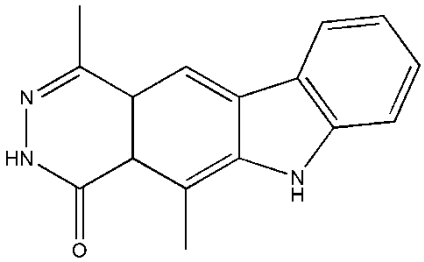
[1]

2. Ewies F. Ewies *et al.*,[20] synthesised some novel pyridazine derivatives[2] and evaluated for its antitumor activity.The invitro cytotoxicity activity was carried out against liver HEPG2 cancer cell lines in comparison to the known anticancer drugs: 5-Flurouracil (5-FU) and Doxorubicin (DOX) using SRB assay. The cytotoxic and growth inhibitory activity of the compound 3a (IC50: 3.92 μg/mL) was very close to that of the 5-Flurouracil reference drug (IC50: 5 μg/mL) against liver carcinoma cell line (HEPG2).



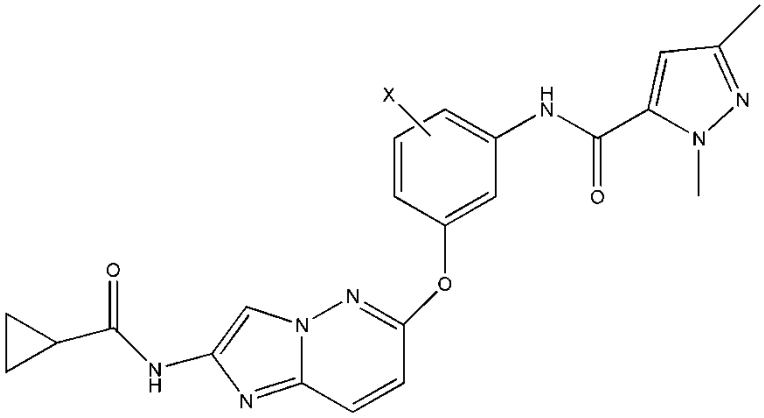
[2]

3. **Mohammad Asif** [1] studied the anticancer potential of various pyridazines[3] and related compounds. Almost all compounds showed remarkable activity against leukemia, non-small cell lung cancer, colon, central nervous system, melanoma, ovarian and breast cancer cell lines.



[3]

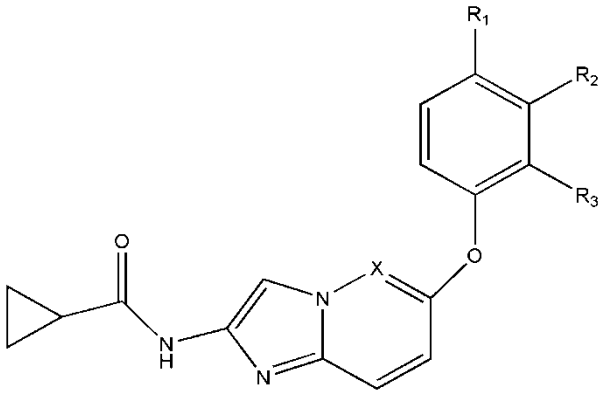
4. Naoki Miyamoto *et al.*,[21] synthesised 2-acylamino-6-phenoxy-imidaz[1,2-b]pyridazine derivatives. Among these, N-[5-({2-[(cyclopropyl carbonyl) amino]imidazo[1,2-b]pyridazine-6yl}oxy)-2-methylphenyl]-1,3-dimethyl-1H-pyrazole-5-carboxamide(**23a,**TAK-593) **[4]** is a highly potent VEGFR2 kinase(PDB Code: 3VO3) inhibitor with an IC50 value of 0.95Nm.The compound shows inhibition of platelet-derived growth factor receptor kinases as well as VEGF receptor kinases in kinase selectivity profiling.



[4]

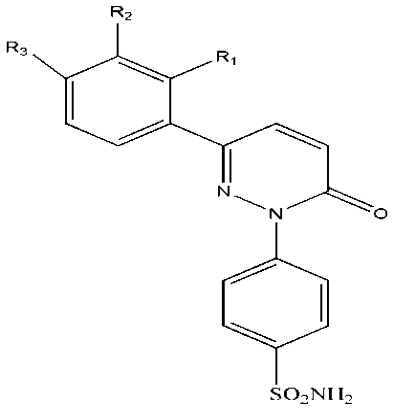
5.Shigemitsu Matsumoto *et al.*,[22] designed and synthesised a novel series of imidazo[1,2-b]pyridazine [5] and imidazo[1,2-a]pyridine derivatives as dual c-

Met and VEGFR2 kinase(PDB code: 3VO3) inhibitors. Compound 26 exhibited antitumour efficacy in vivo in MKN45 and COLO205 mouse xenograft models.



[5]

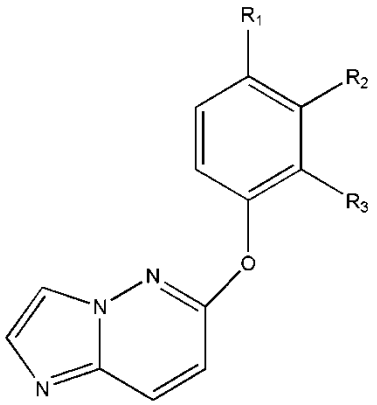
6. **I.G.Rathish *et al.*,**[23] synthesised and studied anticancer activity of some novel 6-aryl-2-(p-sulfamylphenyl)-pyridazin-3(2H)-ones[6]. Among 5 derivatives synthesised ,2h has been selected as lead compound for developing new anticancer agents.



[6]

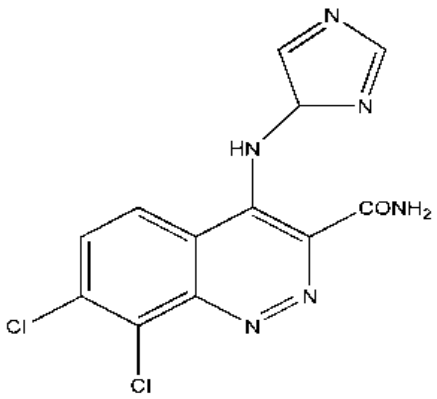
7. Naoki Miyamoto *et al.*,[24] designed and synthesised imidazo[1,2-b]pyridazine derivatives having a benzamide unit and evaluated for its VEGFR2 kinase inhibition.Almost all the tested compounds revealed anti

tumour activity.In which, a strong inhibitory activity was showed by N-[3-(imidazo[1,2-b]pyridazin-6-yloxy)phenyl]-3-(trifluoromethyl)benzamide[7] against VEGFR2 with an IC_{50} value of 7.1Nm.



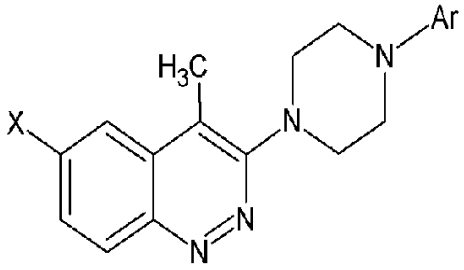
[7]

8. **Mishra Pankaj *et al.,*** [25] synthesised and characterised some cinnoline derivatives and evaluated its activities like anti-hypertensive, antithrombotic, antihistaminic, antileukemic, CNS activity, anti tumor, antibacterial and anti secretory. Halogen substituted Cinnoline imidazole compounds[8] mainly Chloro substituted were showed potent antibacterial, anti-inflammatory and anti-fungal activity than other compounds.However methyl substituted compound also showed more potent antimicrobial activity and anti-inflammatory activity.



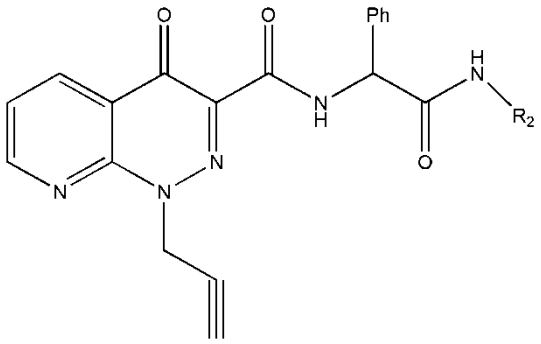
[8]

9. **Eman D. Awad *et al.,*** [26] synthesized series of 6-substituted-4-methyl-3-(4-aryl piperazin-1-yl) cinnolines[9] and the antitumour, antibacterial, and antifungal activity of the newly synthesized compounds were evaluated.



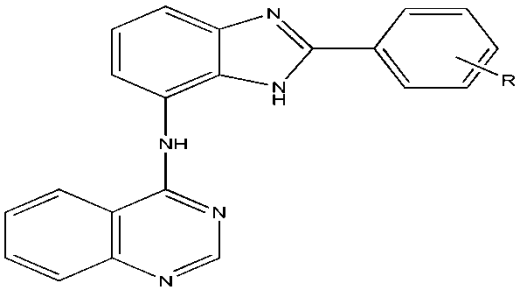
[9]

10. **V. Kumar *et al.,*** [27] synthesised a new series of pyrido[2,3-*c*]pyridazine derivatives[10] and screened for its antitumor activity. Amongst them few of the compounds have exhibited promising cytotoxicity along with good safety index and could be of use in designing new anti-cancer agents.



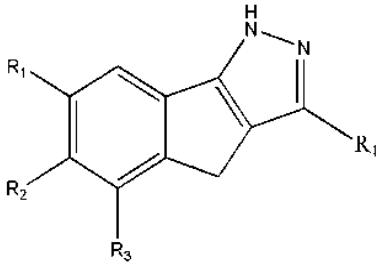
[10]

11. **Lei Shi *et al.,*** **[28]** designed and studied a series of N-(2-phenyl-1H-benzo[d]imidazol-5-yl)quinazolin-4-amine derivatives[11] as dual c-Met and VEGFR-2 inhibitors. Both c-Met and VEGFR-2 are important targets for the treatment of cancers. Among these compounds, 7j exhibited the most potent inhibitory activity against c-Met and VEGFR-2 with IC50 of 0.05 lM and 0.02 lM, respectively. It also showed the highest anticancer activity against the tested cancer cell lines with IC50 of 1.5 lM against MCF-7 and 8.7 lM against Hep-G2. Docking simulation supported the initial pharmacophoric hypothesis and suggested a common mode of interaction at the ATP-binding site of c-Met and VEGFR-2, which demonstrates that as a potential agent for cancer therapy deserving further researching.



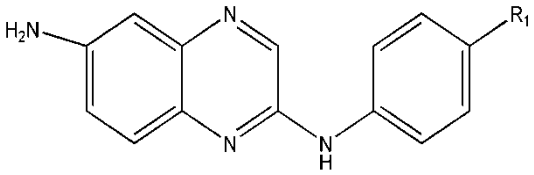
[11]

12. **Huahui Zeng *et al.,*[29]** designed 3D-QSAR modeling and molecular docking study on 1,4-dihydroindeno[1,2-c]pyrazoles [12] as VEGFR-2 kinase inhibitor. By using molecular docking method, binding of VEGFR-2(PDB ID:1Y6A) with its inhibitors were studied. The satisfactory results were obtained suggest that these methods are reasonable for the prediction of new inhibitors and in future drug design.



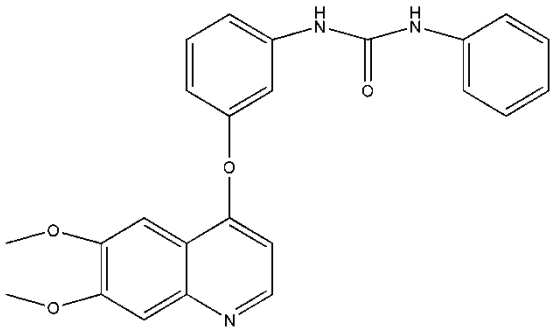
[12]

13. **Mai. I. Shahin [30]** designed and synthesised type-II VEGFR2 inhibitor based on quinoxaline[13] scaffold. The target compounds are biologically evaluated for their inhibitory activity against VEGFR2.



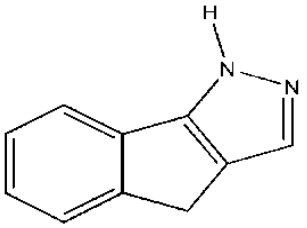
[13]

14.**Vinod G. Ugale *et al.,*[31]** studied pharmacophore modeling for a series of quinoline derivatives[14] as VEGFR2 inhibitors. Docking studies were carried out to analyse drug-receptor interactions and may prove helpful for further lead optimization and virtual screening.



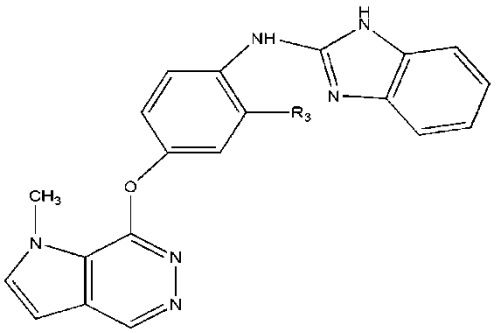
[14]

15. **Rachana R.Desai *et al.*,**[32] studied docking of Vascular Endothelial Growth Factor with phytochemicals for anti-angiogenesis. The molecular docking studies and post processing of docking results were carried out with natural compounds(phytochemicals) as ligand[15] against VEGF in comparison with Bevacizumab an anti-VEGF monoclonal antibody in order to avoid the side effects of older chemotherapy.



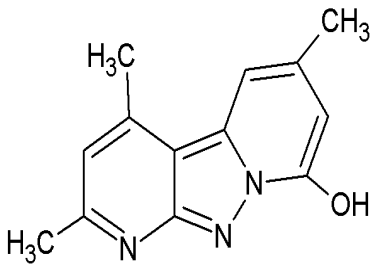
[15]

16. **Yuya Oguro *et al.*,**[33]synthesized a series of pyrrolo[3,2-d]pyrimidine derivatives and evaluated their application as type-II inhibitors of vascular endothelial growth factor receptor 2 (VEGFR2, PDB code: IVR2) kinase.Among these derivatives, compound[16] showed the strongest inhibition of VEGF-stimulated proliferation of human umbilical vein endothelial cells (HUVEC).The co-crystal structure of [17] and VEGFR2 revealed that[17]binds to the inactive form of VEGFR2. The studies reveals that [17] inhibited VEGFR2 kinase with slow dissociation kinetics and also inhibited PDGFR and Tie-2 kinases.



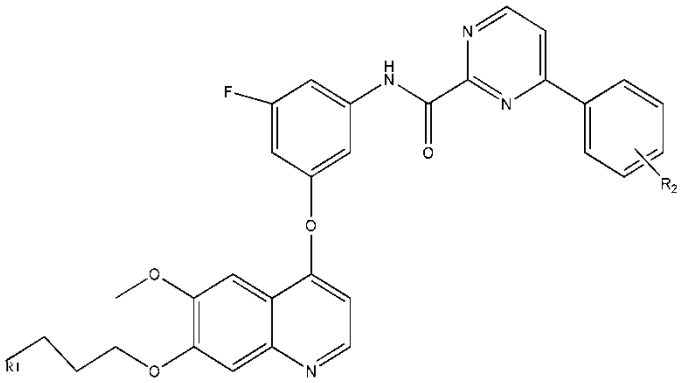
[16]

17. Mosaad Sayed Mohamed *et al.,* [34] synthesized a series of cyanopyridine derivatives, Schiff bases, urea and thiourea derivatives, amide derivatives, pyridopyrazolopyrimidine[17] and pyridopyrazolotriazine. Activities of eleven representative compounds were evaluated against A-549 (lung), HEPG2 (liver) and HCT-116 (colon) cancer cell lines. The findings revealed that some of the synthesized compounds showed remarkable anticancer activities. In addition to synthesis and biological activities, binding features of the potent compound with cyclin dependent kinase was performed using structure based modelling tools.



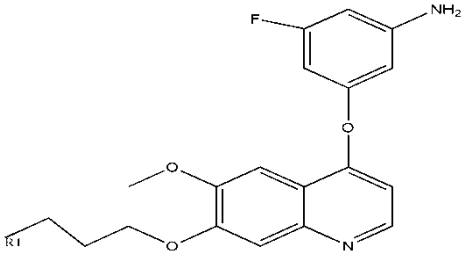
[17]

18. **Qidong Tang *et al.,*** [35] synthesised two series of quinoline derivatives bearing the pyridine/pyrimidine scaffold[18] , and evaluated for their c-Met kinase inhibitory activity and antiproliferative activity against 5 cancer cell lines (HT-29, H460, MKN-45, A549, and U87MG) were evaluated in vitro. Structure activity relationship studies indicated that regulation of the electron density on the pyridine/pyrimidine ring to a proper degree was a key factor in improving the antitumor activity.



[18]

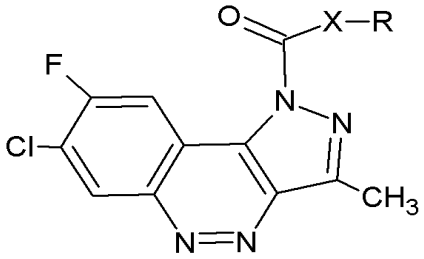
19. **Sai Li *et al.,*** [36] designed and synthesised a series of bisquinoline derivatives connected by 4-oxy-3-fluoroaniline[19] moiety. In-vitro antitumour activities against a panel of five cancer cell lines (H460, HT-29,MKN-45, U87MG, and SMMC-7721) evaluated. An analysis of structureeactivity relationships indicated that an unsubstituted or a halogen-substituted phenyl ring on the 2-arylquinoline-4-carboxamide moiety was favourable for antitumour activity.



[19]

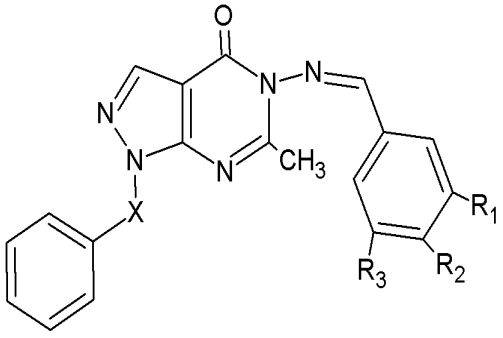
20. **Rajiv Kumar Tonk *et al.,*** [37] synthesized and characterized a series of pyrazolo[4, 3-c] cinnoline derivatives[20] and evaluated for anti-inflammatory and anti-bacterial activity. Test compounds that exhibited good anti-inflammatory activity were further screened for their ulcerogenic and lipid peroxidation activity. Compounds 4d and 4l showed promising anti-inflammatory activity with reduced ulcerogenic and lipid peroxidation activity when compared to naproxen. Docking results of these two compounds with COX-2 (PDB ID: 1CX2) also exhibited a strong binding profile. Among the

test derivatives, displayed significant antibacterial property against gram-negative (Escherichia coli and Pseudomonas aeruginosa) and gram-positive (Staphylococcus aureus) bacteria. However, emerged as the best dual anti-inflammatory antibacterial agent in the present study.



[20]

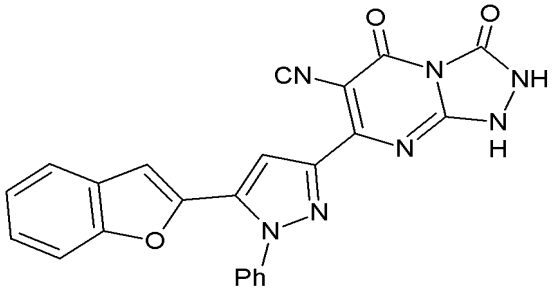
21**. Ghaneya Sayed Hassan *et al.*,**[38]synthesized new series of pyrazolo [3,4-d]and pyrazole hydrazones[21] and evaluated for their antiproliferative activity against human breast adenocarcinoma MCF-7 cell line. Most of the tested compounds exploited potent to moderate growth inhibitory activity to the reference drug cisplatin. The antitumor activity of the new compounds was accompanied by significant increase in the activity of superoxide dismutase with concomitant decrease in the activities of catalase and glutathione peroxidase and reduced glutathione level. Accordingly, the overproduction of hydrogen peroxide, nitric oxide and other free radicals allowed reactive oxygen species (ROS)-mediated tumor cells death, as monitored by reduction in the synthesis of protein and nucleic acids.



[21]

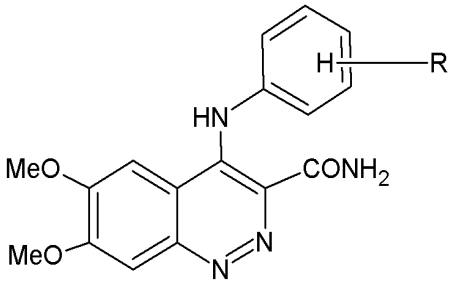
22. **Magdy I. El-zahar *et al.*,** [39] synthesized a new series of (benzofuran-2-yl)-1-phenyl-1*H*-pyrazol-4-yl) pyrimidine derivatives[22]. Some docking

studies of the newly prepared compounds as thymidylate synthase inhibitors have been done. The cytotoxic activity of some of the prepared compounds as a representative examples was evaluated against HEPG2 (human liver carcinoma cell line) in comparison with 5-fluorouracil (5-Fu).



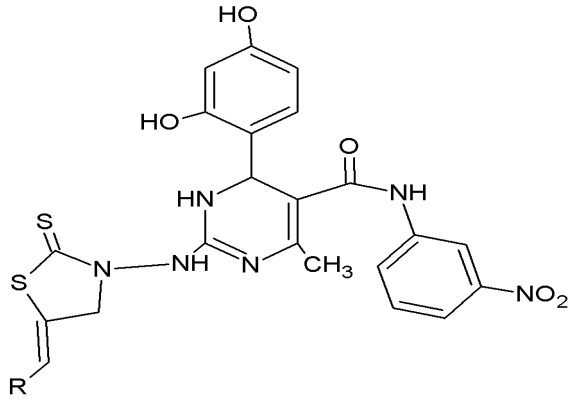
[22]

23. **David A. Scott *et al.*,**[40] 3-Amido-4-anilinocinnolines[23] have been identified as potent and highly selective inhibitors of CSF-1R. The synthesis and SAR of these compounds is reported, along with some physical property, pharmacokinetic and kinase selectivity data.



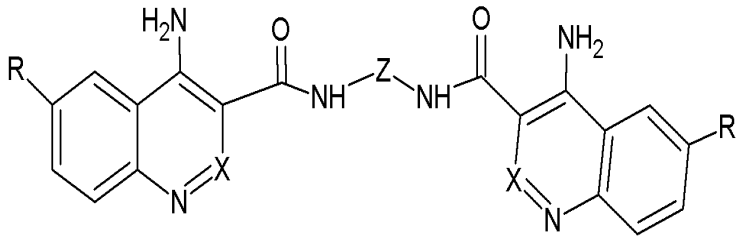
[23]

24. **Diaa A. Ibrahim *et al.*,**[41]designed and synthesized novel derivatives of 2, 4,5,6-tetrasubstituted pyrimidine cyclin-dependent kinase[24] (CDK2) inhibitors. A library of proposed pyrimidine derivatives was built and by using pharmacophore and docking techniques selections were made. The newly synthesized compounds showed potent and selective CDK2 inhibitory activities and inhibited in-vitro cellular proliferation in cultured human tumour cells.



[24]

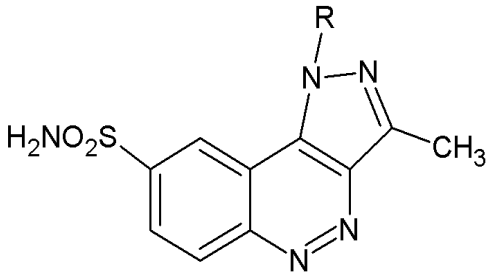
25. **Marta Szumilak *et al.*,**[42] synthesised new polyamine derivatives containing dimeric quinoline, cinnoline[25] and phthalimide moieties. The new compounds were obtained according to known procedures. Their biological activity was assessed in vitro in a highly aggressive melanoma cell line A375. Polyamine diimides containing phthalimide moieties demonstrated no inhibitory activities against melanoma cells. Quinoline diamides were more efficient than cinnoline ones. Mainly cytostatic activity exerted as altered cell cycle profiles was observed at the concentrations causing about 50% reduction of adherent cell proliferation. Based on their structure as well as their biological activity, we assume that some of the newly synthesized compounds may act as DNA bisintercalators. This study might be useful for further designing and developing anticancer drugs with potent activities.



[25]

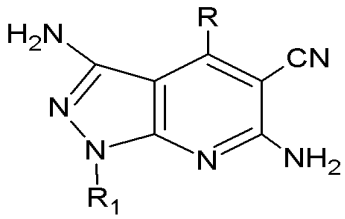
26. T.V.Yuvaraj *et al.*,[43]synthesised a series of 3'-methyl-6-sulphamido-1'substituted-pyrazolo [4,3-c] cinnoline derivatives[26]. The compounds were characterized by analytical techniques like TLC, UV, IR, NMR Spectral

studies. The compounds were screened for antimicrobial activity against bacterial organisms like Escherichia coli, Klebsiella aurogeniosea, Micrococcus luteus, Bacillus cereus and fungal organism like Candida albicans.



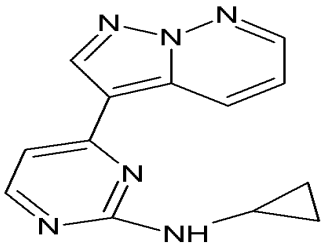
[26]

27. **Mourad Chioua *et al.*,**[44]synthesised and biologically evaluated a number of differently substituted 3,6-diamino-1H-pyrazolo[3,4-b]pyridine derivatives[27]. From the inhibition results on a selection of disease-relevant protein kinases they observed that 3,6-diamino- 4-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile constitutes a potential new and simple lead compound in the search of drugs for the treatment of Alzheimer's disease.



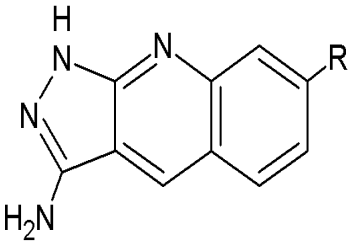
[27]

28. **Kirk L. Stevens *et al.*,**[45] synthesized and identified a novel series of pyrazolo [1,5-b] pyridazines[28] as cyclin dependant kinase inhibitors potentially useful for the treatment of solid tumours. Modification of the hinge-binding amine or the C (2) and C (6) substitutions on the pyrazolopyridazine core provided potent inhibitors of CDK4 and demonstrated enzyme selectivity against VEGFR-2 and GSK3β.



[28]

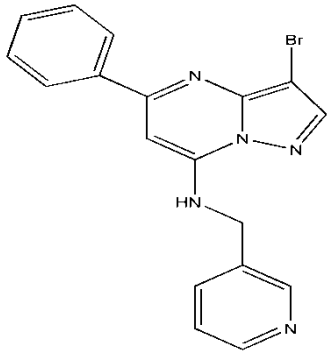
29.**Jamal M. Arif *et al.,*** [46]screened newly synthesized antiviral aminopyrazoloquinoline derivatives[29] for cytotoxic potential in human normal and breast cancer cell lines using apoptosis as biomarker. These derivatives and the well known antiviral drug, acyclovir, were incubated with the normal (MCF-10A, MCF-12A) and cancer (MCF-7, MDA-MB-231) cell lines. Both the parent compounds and their sugar derivatives were found to be differentially cytotoxic in various cell lines. Their study suggested that the newly synthesized antiviral compounds have an associated risk of being cytotoxic compared to the acyclovir.



[29]

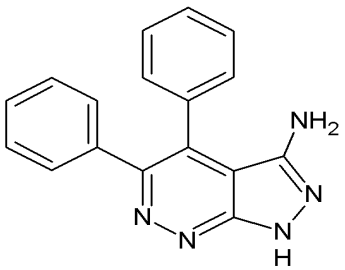
30. **Thierry O. Fischmann *et al.,*** [47] discovered CDK2 inhibitors containing the related bicyclic heterocycles imidazopyrazines and pyrazolopyrimidines[30] through high-throughput screening. Crystal structures of inhibitors with these bicyclic cores and two more related ones show that all but one have a common binding mode featuring two hydrogen bonds (H-bonds) to the backbone of the kinase hinge region. Even though ab initio computations indicated that the imidazopyrazine core would bind more tightly to the hinge, pyrazolopyrimidines gain an advantage in potency through

participation of N4 in a H-bond network involving two catalytic residues and bridging water molecules. Further insight into inhibitor/CDK2 interactions was gained from analysis of additional crystal structures.



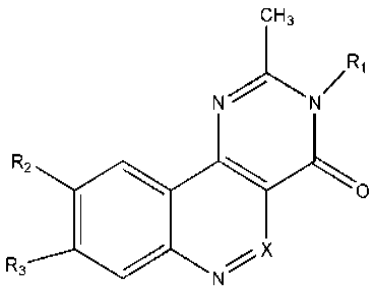
[30]

31. Miguel F. Brana *et al.*,[48]identified Pyrazolopyridazine[31] in a high-throughput screening carried out by BASF Bioresearch Corp. as a potent inhibitor of CDK1/cyclin B and shown to have selectivity for the CDK family. Analogues of the lead compound were synthesized and their antitumour activities have been tested. A molecular model of the complex between the lead compound and the CDK2 ATP binding site has been built using a combination of conformational search and automated docking techniques.



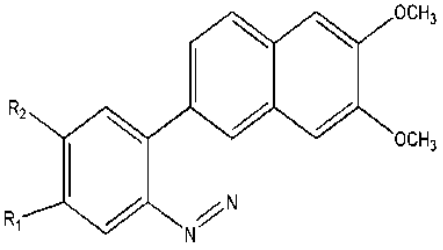
[31]

32.**Wieslawa Lewgowd *et al.*,**[49] synthesised pyrimido[5,4-c]cinnoline [32] and pyrimido[5,4-c]quinoline derivatives and determined its cytotoxicity on the two human leukemia cell lines, the promyelocytic HL-60 and the lymphoblastic NALM-6.The continous exposed cell viability was found out by the tryptan-blue exclusion assay. IC_{50} value suggested that the HL-60 leukemia cells are more resistant to toxic action of tested compounds.All compounds exerted moderate cytotoxic activity.



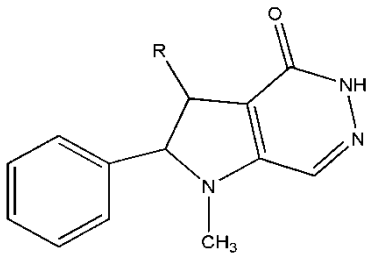
[32]

33. **Younong Yu *et al.,*** [50] synthesised several substituted dibenzo[c,h] cinnolines [33] and evaluated for their potential to target topoisomerase I and for their relative cytotoxicity activity as in anticancer agents.With regards to topoisomerase I-targeting activity and cytotoxicity 2,3-Dimethoxy-8,9-methylenedioxybenzo[i]phenanthridine was one of the more potent benzo[i]phenanthridine derivative.These results indicate that substituted dibenzo[c,h]cinnolines can exhibit potent topoisomerase I targeting activity and are capable of overcoming the multi-drug resistance associated with this efflux transporter.



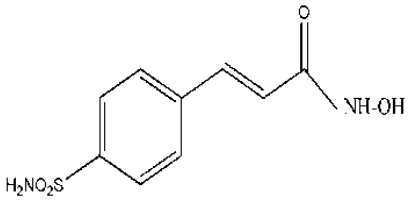
[33]

34.**Gabriele Murineddu *et al.,*** [51] synthesised and evaluated in vitro cytotoxicity activity of Pyrrole[2,3-d]pyridazine-4-one derivative[34] against 60 human tumor cell lines derived from nine cancer cell types. Among them, the most potent compound 3 showed significant cell line cytotoxicity, particularly against the renal cancer subpanel and displayed significant potency against MOLT-4, SR (leukemia), NCI-H460 (non-small cell lung), HCT-116 (colon), and SF-295 (CNS) cancer cells, respectively.



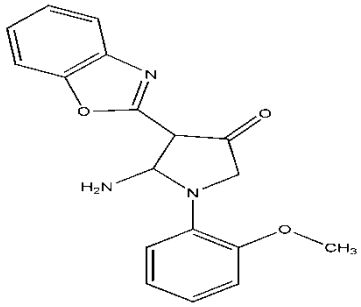
[34]

35. **Neetinkumar D. Reddy *et al.*,**[52] evaluated invitro and invivo studies of novel cinnamyl sulphonamide hydroxamate derivative[35] against colon adenocarcinoma.1,2-Dimethyl hydrazine(DMH) was used to produce experimental colon adenocarcinoma in Wistar rats. 5-FU and NMJ-2(100mg/kg p.o. and 10mg/kg i.p. once daily for 21 days, respectively) were administered to the respective groups.Both therapy showed significant reduced ACFs, adenocarcinoma count, TNF-a,IL-6 and nitrate in colonic tissue.The results indicates that NMJ-2 has potent anti-cancer activity through HDAC enzyme inhibition.Thus, the effectiveness of these molecules were evaluated by whole cell HDAC assay in HCT 116 cell line.



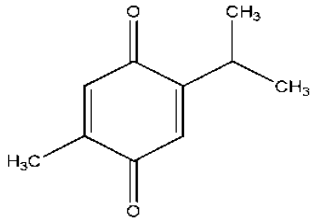
[35]

36. H.M.Kuznietsova *et al.*,[53] evaluated the comparative effects of cytostatic compound dihydropyrrol derivative(D_1)[36] and 5-Fluorouracil(5-FU) on the normal colonic mucosa of tumor-bearing rats and also correlated the proliferation of normal colonic mucosa and tumor growth parameters.DMH carcinogenic model was used.D_1 manifests the same antitumor activity with less toxicity compared to 5-FU that allows it to meaned as an anticancer agent.



[36]

37. Wasfi Asfour *et al.*,[54] studied the chemopreventive efficacy of Thymoquinone(TQ)[37] against DMH induced colon carcinogenesis in the rat model.A two-phase study was used to evaluate its potential impact on tumor progression and invasion in vivo.TQ treatment demonstrated a negative impact on vascular endothelial growth factor(VEGF) production in tumor-bearing rats which results in suppressed cellular proliferation.The results could provide an effective approach in the primary prevention of colon cancer in humans in the next future.



[37]

38. **Aranya Manosroi *et al.*,[55]** evaluated the invitro anti-cancer activities of Job's tears(Coix lachrymal-jobi Linn.) extracts on human colon adenocarcinoma cell line(HT-29) by sulforhodamine B (SRB)assay.

3. AIM AND OBJECTIVES

Cancer is a group of diseases characterised by a high proliferative index and the spread of aberrant cells from their site of origin. A combination of surgery and radiotherapy with chemotherapy is the clinical therapeutic treatment for cancer. Recent chemotherapy consists of cytotoxic agents and anti-hormonal drugs, which reduce the proliferation of the tumors. The use of anticancer drugs is complicated by systemic toxicity, generally in the bone marrow, hair and gastrointestinal tract, and also by development of resistance. As a result, the search for new chemical structures with broader therapeutic windows and acceptable resistance profiles is being vigorously pursued. Discovering new anticancer agents requires continued commitment to the demanding tasks remains critically important. **[1]**

The first step for the discovery of anticancer drugs should be focused on the use of methodologies which can provide a rationalization of the serendipitous process of organic synthesis in Medicinal Chemistry and Drug Design. In this sense, the modern science has the strong support of novel paradigms which have been essential for the discovery of new chemotherapies for dissimilar diseases: Molecular Docking and Computer Aided Drug Design (CADD).[15]

More recently, pharmaceutical attention has been focussed on the proteins that drive and control cell cycle progression.The significance of these potential drug targets is clearly evidenced by the high incidence of alterations in the genes that code for these proteins in tumours. One such protein is **Vascular endothelial growth factor receptor2** which are proved to be viable target for development of effective and safe anti- cancer drugs.

Angiogenesis is the genesis of new blood vessels which involves the migration ,growth and differentiation of endothelial cells, which line inside the wall of the blood vessels and is controlled by chemical signals in the body which stimulates the repair and formation of blood vessels. The process of angiogenesis has an important role in the growth and metastasis of cancer. A blood supply is essential for cancer cells to grow beyond a few millimetres in size and also stimulate the nearby normal cells to form angiogenesis signaling molecules.Without a blood supply, cancer cells cannot grow beyond a limit , hence the scientists were trying to find solutions to block tumor angiogenesis. Angiogenic inhibitors were studied and

identified with the idea that these molecules will prevent or slow down the cancer growth.[56]

There are several regulators of angiogenesis, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor(PDGF),basic fibroblast growth factor(bFGF), and angiopoietin. The VEGF signaling pathway through the tyrosine kinases receptor VEGFR-1 and VEGFR-2 are critical regulators of angiogenesis.The VEGF receptor protein-tyrosine kinases consist of an extracellular component with 7 immunoglobulin-like domains, a juxtamembrane segment,a single transmembrane segment,an intracellular protein-tyrosine kinase domain that has an insert of nearly 70 amino acid residues, and a carboxyterminal domainal tail.A conformational change will occurs after the binding of VEGF to VEGFR2 and will leads to receptor dimerization ,which is followed by the autophosphorylation of tyrosine residues present in the intracellular kinase domain.Vascular endothelial growth factor (VEGF) signaling through VEGF receptor-2 (VEGFR2) has been shown to play a major role in the regulation of tumour angiogenesis **(fig. 10)**. Therefore, inhibition of **VEGFR-2** has become an attractive target for the development of novel anticancer agents.[24]

Figure 10: represents VEGF/VEGFR Role in Tumor Angiogenesis

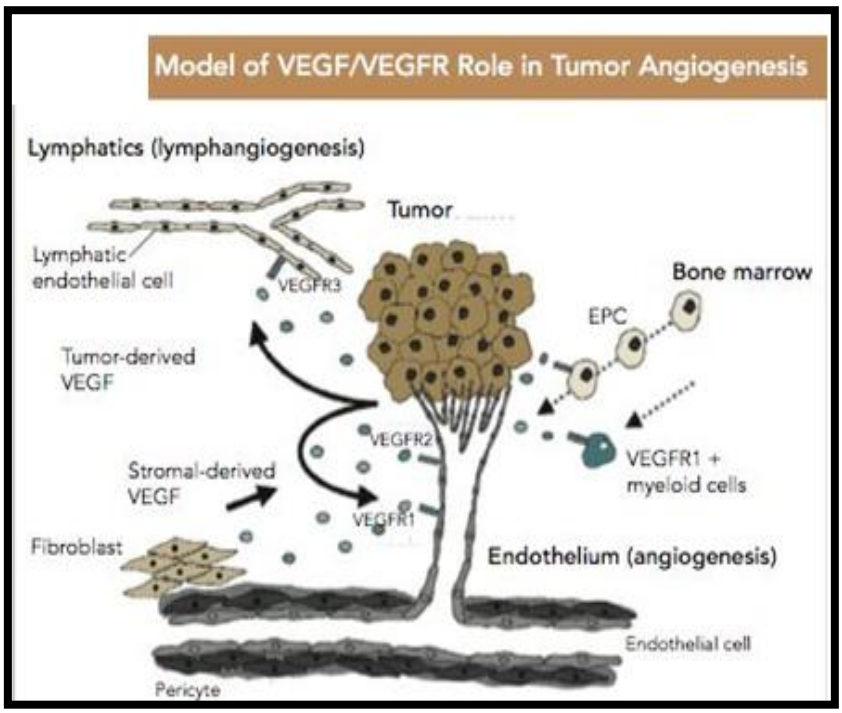
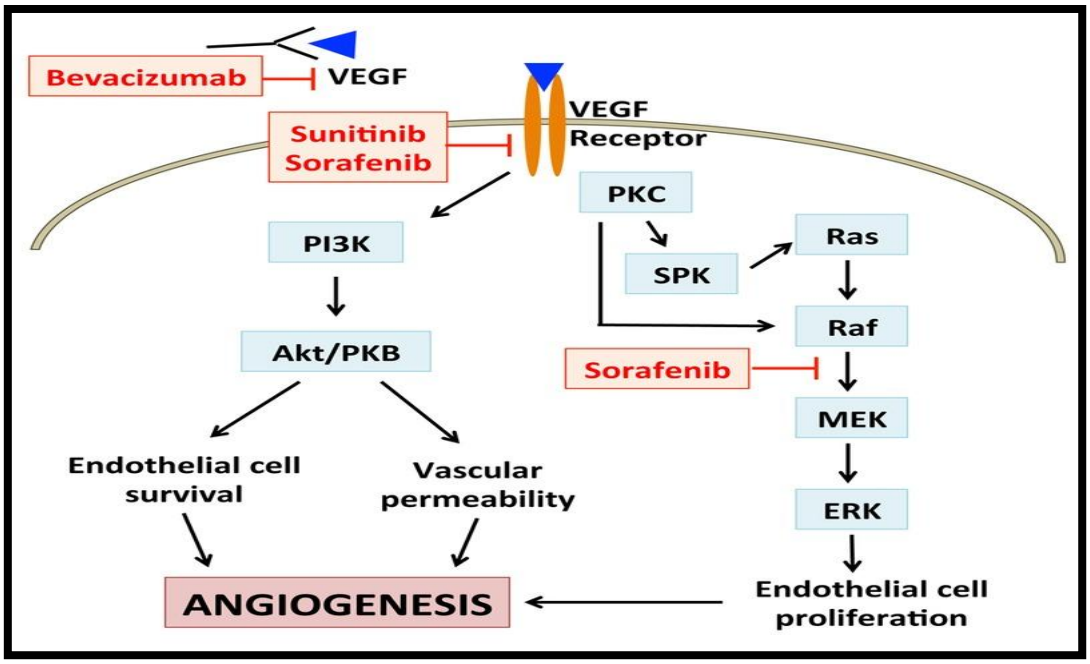
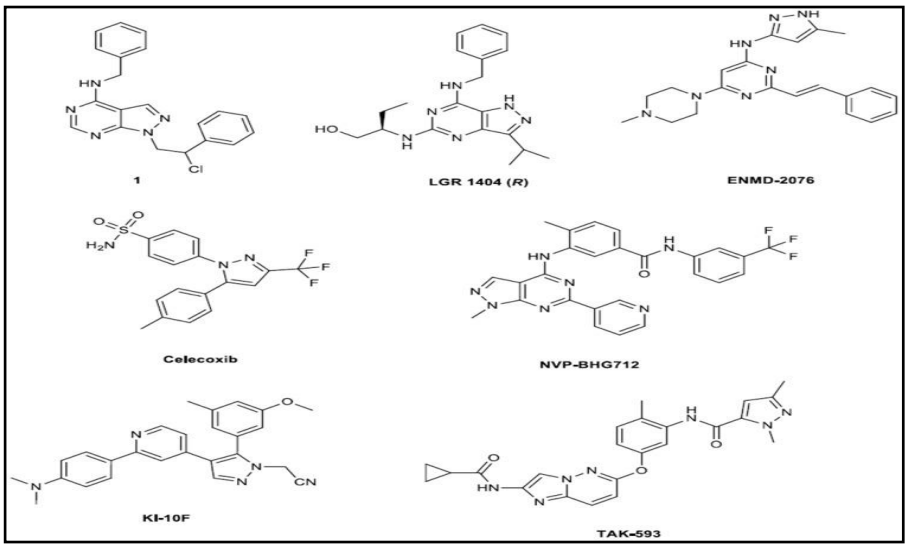


Figure 11: Anticancer Drugs Targeting VEGF Pathway



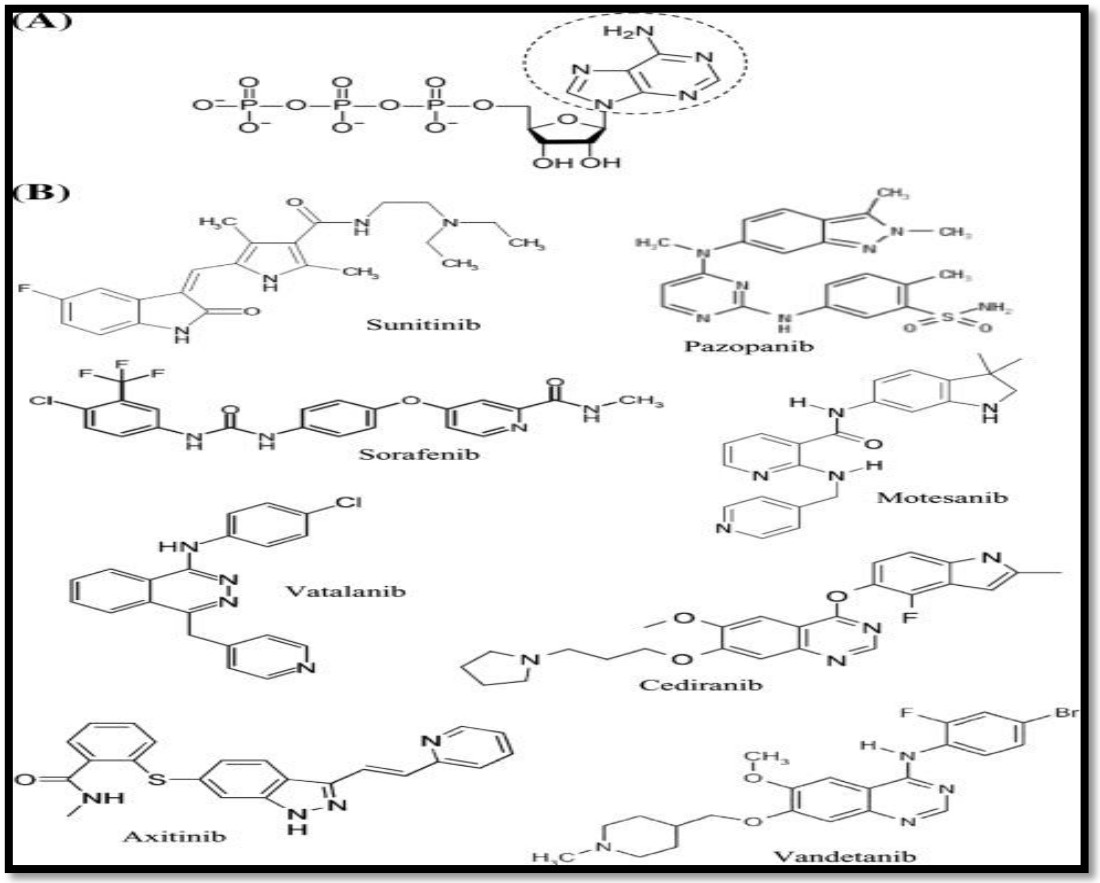
Many anticancer drugs that are available in the market and some under clinical trials have structural similarity with nucleotides and acts as competitive inhibitors of nucleotides that binds with tyrosine kinases receptor like VEGFR and other tyrosine kinases and thus retards tumour growth.[56]

Competitive Inhibitors Of Nucleotides



The search for small-molecule inhibitors of VEGFR2s has already led to the discovery of several classes of compounds with high structural diversity. Most VEGFR2 inhibitors share common properties and they act by competing with ATP for binding in the kinase ATP binding site and thus retards tumour growth.[56]

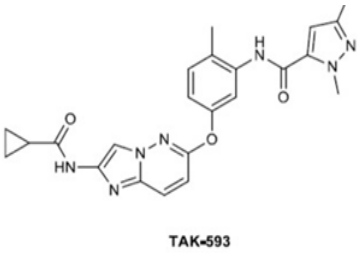
Structure: (A)- ATP, (B)-Anti cancer drugs acting as competitive inhibitors of ATP by binding with VEGFR.



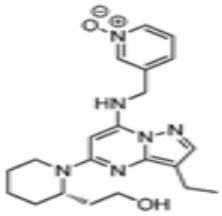
Drugs that inhibit VEGF from working can be used to treat some colon or rectal cancers. These include:

- **Bevacizumab (Avastin)**
- **Ramucirumab (Cyramza)**
- **Ziv-aflibercept (Zaltrap)**
- **sorafenib, sunitinib, pazopanib, Lapatinib and axitinib** are the drugs being evaluated in clinical trials.

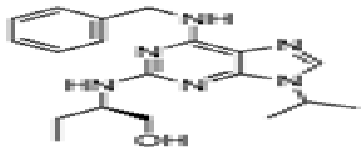
Pyrazolo pyridazine have structural similarity with many purine nucleotides [pyrimidine and imidazole] like ATP which are helpful in the cell growth and thus Pyrazolo pyridazine can also compete with nucleotides for binding with the receptor proteins and can retard the growth of abnormal cells.



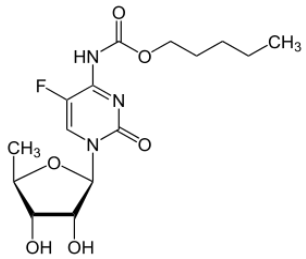
TAK-593



Dinaciclib
(SCH-727965)

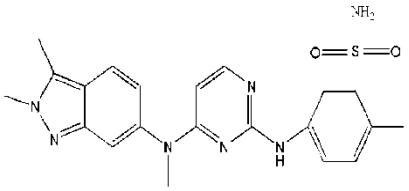


(R)-Roscovitine

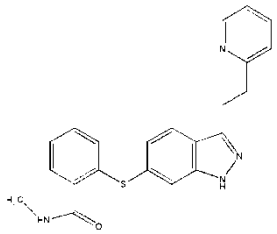


Capecitabine

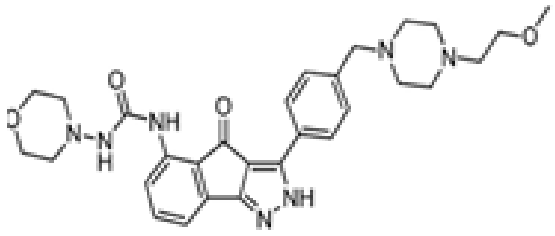
Pyrazole is a versatile nucleus with various biological activities, and many anti cancer drugs have the pyrazole moiety.[14]



1**. Pazopanib**



2. Axitinib

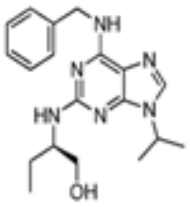


RGB-286638

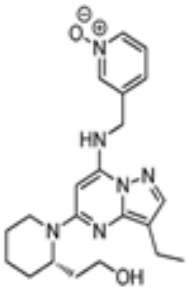
A huge number of pyridazine derivatives attract significant attention due to the recent reports of its antitumor activity. Some of the pyridazinone derivatives bearing different moieties were exhibited excellent anticancer activity toward human cancer cell lines. They showed remarkable activity against leukemia,

non-small cell lung cancer, colon, central nervous system, melanoma, ovarian and breast cancer cell lines. There are a variety of mechanisms of their antitumor action, including carbonic anhydrase inhibition, disruption of microtubule assembly, cell cycle arrest in the G1 phase, functional suppression of the transcriptional activator and angiogenesis inhibition. Currently, some of these derivatives are being evaluated in clinical trials, with much optimism that may lead to novel anticancer drugs, which is devoid of the side effects of the recently available anticancer drugs.[1]

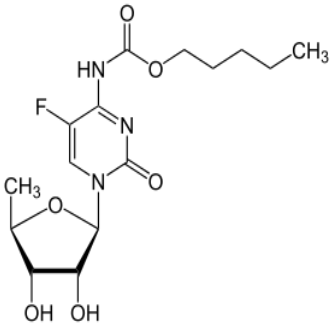
- Pyrazolo pyridazine is isosteric with many nucleosides which are acting as anti tumour drugs which are also available in the market.



(R)-Roscovitine

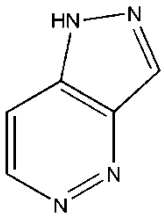


Dinaciclib
(SCH-727965)



Capecitabine

- Moreover, the pyrazole and pyridazine nucleus have a broad spectrum of biological activities such as anti-inflammatory, antioxidant, ACE inhibition, antitumour, antitubercular, antiviral, antidepressant, anticonvulsant, antifungal and antibacterial activities.
- The therapeutic importances of this nucleus as antitumour agents have prompted us to select this nucleus.



- The emphasis has been put on the molecular modulation specifically modifying different substituents and its positions in the aromatic system.

- Therefore efforts were taken to design and develop Pyrazolo benzpyridazine derivatives whose basic nucleus will be a structural analog with nucleosides involved in cell cycle and can compete with it and to study its effect against cancer cells in order to get potent anti-tumour agents.

- Hence the aim and objective of study was to design the lead molecules based on literature and to study its binding energies and docking interactions with specific protein VEGFR family and to synthesis Pyrazolo benzpyridazine derivatives and its *in vitro* and *in vivo* screening for anticancer activity.

4. PLAN OF WORK

Target selection

↓

Selection and design of Ligand

↓

Lead optimisation by Molinspiration Software

↓

Molecular Docking studies by Glide 10.1 Software

↓

Synthesis, characterisation and spectral analysis.

↓

In-vitro antioxidant study of synthesised compounds by DPPH and ABTS radical scavenging assay.

↓

In vitro cell line (HT 29) assay by MTT method.

↓

Acute toxicity studies according to OECD Guideline 423.

↓

In vivo animal studies.

1. Estimation of Tumerogenesis
2. Estimation of Haematological Parameters like RBC,WBC and Haemoglobin
3. Estimation of Serum Biochemical Parameters like SGOT, SGPT, ALP, Creatinine, total protein.
4. In vivo antioxidant Study includes catalase, SOD, GPx, GSH, Lipid peroxidation.

5. Estimation of Enzymes involved in Citric acid cycle[TCA]
6. Histopathological analysis
7. Immunohistochemical reactivity

Statistical analysis

Results and discussion

5. METHODOLOGY

5.1 TARGET SELECTION

The accumulation of genetic and biological information from solid tumours is providing additional targets that have the potential of yielding drugs with broad activity and less toxicity than current therapies. Because of genomic stability, the endothelial cells are found to be an ultimate target for therapies focussed against cancer cells.[45]

Angiogenesis is the process in which angiogenic endothelial cells will undergo a series of events that includes the secretion of metallo-proteases and other matrix-degrading enzymes, cell migration into the newly created space, endothelial cell division and proliferation, including new capillary blood vessel formation from pre-existing vasculature. There are several regulators of angiogenesis, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor(PDGF),basic fibroblast growth factor(bFGF), and angiopoietin. The VEGF signaling pathway through the tyrosine kinases receptor VEGFR-1 and VEGFR-2 are critical regulators of angiogenesis. The VEGF receptor protein-tyrosine kinases consist of an extracellular component with 7 immunoglobulin-like domains, a juxtamembrane segment, a single transmembrane segment, an intracellular protein-tyrosine kinase domain that has an insert of nearly 70 amino acid residues, and a carboxyterminal domainal tail. The reaction catalysed by these enzymes include:

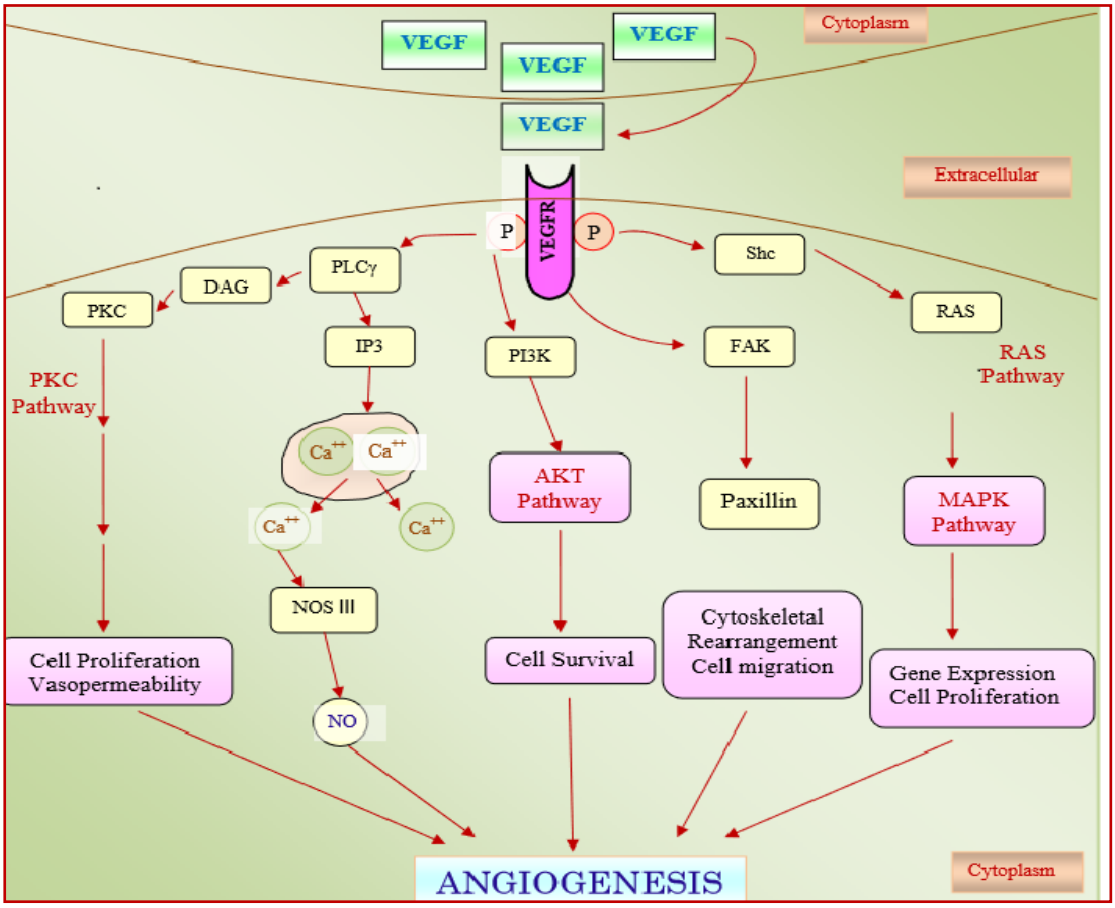
$$MgATP^{1-} + \text{protein-OH} \longrightarrow \text{Protein-}OPO_3^{2-} + MgADP + H^+$$

Where –OH is a tyrosyl hydroxyl group.

A conformational change will occurs after the binding of VEGF to VEGFR2 and will leads to receptor dimerization ,which is followed by the autophosphorylation of tyrosine residues present in the intracellular kinase domain**(fig.12)**.[57] The VEGF/VEGFR2 signaling pathway stimulates the cancer development by supplying oxygen and nutrients for the growing tumour cells, hence it plays an important role in tumour angiogenesis. VEGF is overexpressed in various classes of human tumours and its levels can be correlated with poor prognosis and clinical stage in patients with

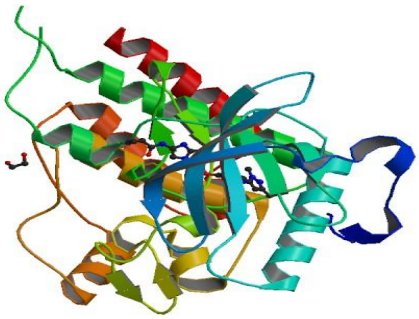
solid tumours . Therefore, VEGF/VEGFR2 signaling can de regarded as an potential target in the treatment of cancer. In fact, several VEGFR2 inhibitors including sorafenib, sunitinib, pazopanib and axitinib are being evaluated in clinical trials. VEGFR2 inhibitors have garnered attention recently for their potential as anticancer therapeutics.**VEGFR2 was selected as the target due to the potential as good anti-cancer target.**[24]

Figure 12: Biochemical conversion



Various protein structures of VEGFR2 in complex with different ligands are available in RCBS protein data bank, out of which 3VO3 has been selected because of the structural similarities of the ligand-N-[3-({2-[(cyclopropylcarbonyl) amino] imidazo[1,2-b]pyridazin-6-yl}oxy)phenyl]-1,3-dimethyl-1H-pyrazole-5-carboxamide in complex with 3VO3 and the designed molecules and is shown in fig.13.[21]

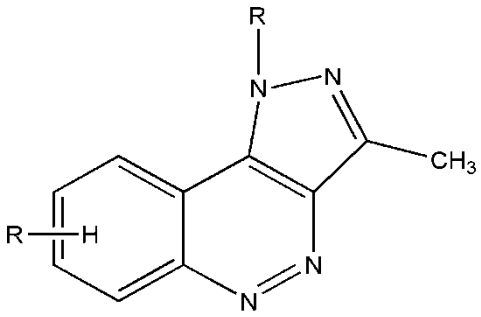
Figure 13: 3VO3 protein in complex with ligand



5.2 *IN SILICO STUDIES*

5.2.1 DRUG DESIGN

Lead molecule designed – Among several classes of potential VEGFR2 inhibitors, focus was made on Pyrazolo benzpyridazine derivatives due to its structural similarity with the natural ligand ATP and with structures of many known biologically active VEGFR2 inhibitors. Hence various Pyrazolo benzpyridazine were designed and emphasis was given on the molecular modulation specifically modifying different substituents and its positions in the aromatic system.



Pyrazolo benzpyridazine

5.2.2 LEAD OPTIMISATION[58]

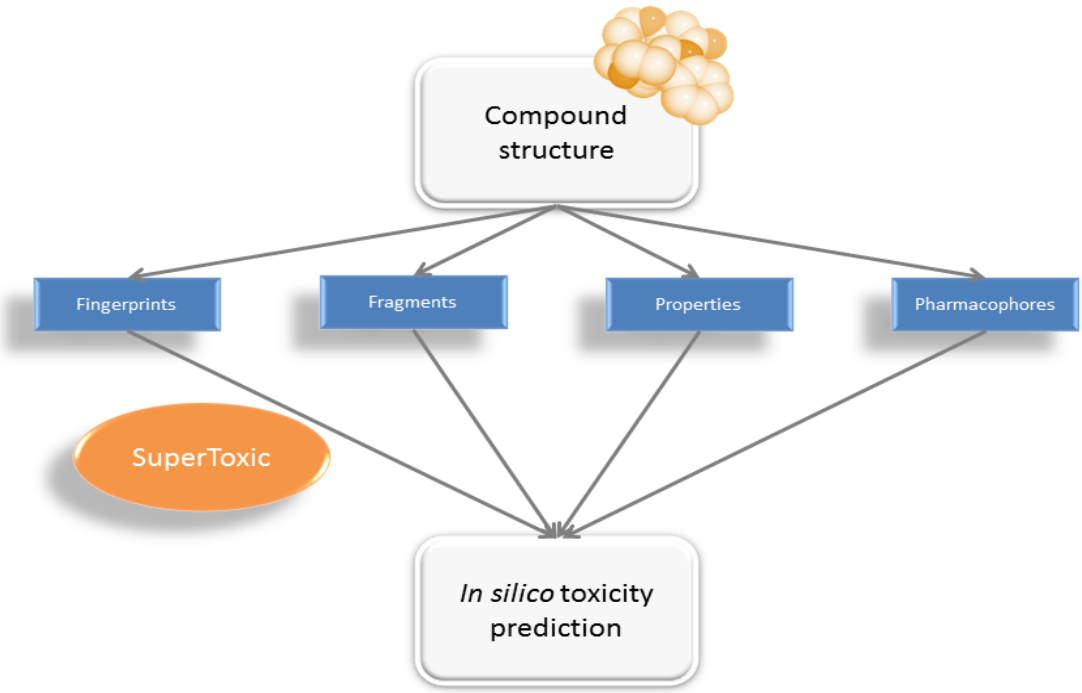
Lead optimization was done through Molinspiration server for the purpose of calculation of logP. The structure drawn in was subjected to calculate the drug likeness score. Molinspiration Property Calculator which allows easy interactive calculation of molecular properties, as well as generation of data tables which may be used for structure-activity QSAR studies.

5.2.3 PREDICTION OF ACUTE TOXICITY[17]

Acute toxicity testing involves an assessment of the general toxic effects of a substance that result either from a single exposure or from multiple exposures in a short period of time (usually less than 24 hours) and these adverse effects should occur within 14 days of the administration of the substance .

Many acute toxicity studies have been conducted solely for the purpose of determining the LD_{50} in mg/kg body weight. The LD_{50} is the median lethal dose meaning the dose at which 50% of test subjects die upon exposure to a compound.

Figure 14: *In silico* toxicity prediction



- PROTOX is a web server for the prediction of oral toxicities of small molecules in rodents, an important part of the drug design development process.
- Computational toxicity estimations are not only faster than the determination of toxic doses in animals, but can also help to reduce the amount of animal experiments.

- PROTOX is based on chemical similarities between compounds with known toxic effects and the presence of toxic fragments.[17]
- GUSAR software was developed to create QSAR/QSPR models on the basis of the appropriate training sets represented as SD file contained data about chemical structures and endpoint in quantitative terms.[18]

5.2.4 DOCKING STUDIES

Glide(grid-based ligand docking with energetics) has been designed to perform as close to an exhaustive search of the positional, orientational, and conformational space available to the ligand as is feasible while retaining sufficient computational speed to screen large libraries.Glide uses a series of hierarchical filters to search for possible locations of the ligand in the active-site region of the receptor. The shape and properties of the receptor are represented on a grid by different sets of fields that provide progressively more accurate scoring of the ligand pose. Docking studies of designed compounds were carried out using GLIDE (Grid-based Ligand Docking with Energetics) module version 10.1, Schrodinger, LLC, New York, NY, 2015. The software package running on multi-processor Linux PC. GLIDE has previously been validated and applied successfully to predict the binding orientation of many ligands.

5.2.4.1 DOCKING METHODOLOGY

The steps involved in docking are as follows:

- **Ligand Preparation:** The 2D structures of the designed molecules were constructed using Glide 2D sketcher and then it was created in 3D structure format. The LigPrep a utility in Schrodinger software suite that combines tools for generating 3D structures.
- **Preparation of protein:** The X-ray crystal structures of the proteins 3VO3 were obtained from the RCSB protein data bank (http://www.rcsb.org/pdb). After selection, the 3-dimensional structures of the protein it was analysed for potential binding pockets and then prepared by docking software Schrödinger-Maestro 10.1. Usually the PDB structure consists of metal ions, cofactors, can contain waters and has no information on bond orders, formal atomic charges and misaligned terminal amide groups Ionization and tautomeric states. In order to acquire accurate energy evaluation the bond orders and ionization

states to be properly assigned, side chains to be reoriented and steric clashes to be relieved as Glide calculations are based on all-atom force field. The missing side-chain atoms were added manually and the proteins were preprocessed separately by deleting the substrate cofactor as well as the crystallographically observed water molecules (water without H bonds), correcting the mistakes in PDB file, optimizing hydrogen bonds. After assigning formal charge with bond orders and protonation state finally energy minimization was done.

- **Receptor grid generation:** Receptor grid generation requires a prepared structure: an all atom structure with appropriate bond order and formal charges. Glide searches for favourable interaction between one or more ligand molecules and a receptor molecule, usually a protein. The shape and properties of the receptor are represented on a grid by several different sets of field that provide progressively more accurate scoring of the ligand poses. The option in each tab of the Receptor Grid Generation panel allow defining the receptor structure by excluding any co-crystallized ligand that may be present, determine the position and size of the active site as it will be represented by receptor grid, and set up Glide constraints. A grid area was generated around the binding site of the receptor.
- **Ligand docking:** This is carried out using GLIDE DOCK. Glide searches for favourable interaction between one or more ligand molecule and a receptor molecule, usually a protein. Each ligand acts a single molecule, while the receptor may include more than one molecule, e.g., a protein and a cofactor. Glide a run in rigid or flexible docking mode; the latter automatically generated conformation for each input ligand. The combination of position and orientation of a ligand relative to the receptor, along with it conformation in flexible docking, is referred to as a ligand poses. The ligand poses that Glide generate pass through a series of hierarchical filter that evaluate the ligand to the defined active site, and examine the complimentarily of ligand-receptor interaction using a grid-based method patterned after the empirical ChemScore function. Poses that passed the initial screen entered the final stage of the algorithm, which involve evaluation and minimization of a grid approximation to the OPLS-2005 non bonded ligand-receptor interaction energy. Final scoring is then carried out on the energy-minimized poses.

- **Glide Extra-Precision Mode (XP) –** The extra-precision (XP) mode of Glide combines a powerful sampling protocol with the use of a custom scoring function designed to identify ligand poses that would be expe8cted to have unfavourable energies, based on well- known principles of physical chemistry. The presumption is that only active compounds will have available poses that avoid theses penalties and also receive favourable scores for appropriate hydrophobic contact between the protein and the ligand, hydrogen-bonding interactions, and so on. The chief purposes of the XP method are to weed out false positives and to provide a better correlation between good poses and good scores. Extra-precision mode is a refinement tool designed for use only on good ligand poses. Finally, the minimized poses are re-scored using Schrodinger's proprietary *Glide Score*s coring function. Glide Score is based on Chem Score, but includes a steric-clash term and adds buried polar terms devised by Schrodinger to penalize electrostatic mismatches:

Glide Score = 0.065*vdW + 0.130*Coul + Lipo + Hbond + Metal + BuryP + RotB +Site

Where, vdW: - Van der Waal energy; Coul: - Coulomb energy; Lipo: - Lipophilic contact term, H Bond: - Hydrogen-bonding term,Metal: - Metal-binding term, BuryP: - Penalty for buried polar groups, Rot B: - Penalty for freezing rotatable bonds, Site: - Polar interactions at the active site; and the coefficients of vdW and Coul are: - a = 0.065, b = 0.1

5.2.4.2 DOCKING PROCEDURE

The computational modelling studies relied upon the GLIDE (Grid-based Ligand Docking from Energetics) program (Glide, version 10.1, Schrodinger, LLC New York, 2015) for the docking simulations. These simulations were performed using the X-ray crystal structure of the human VEGFR2 (PDB ID: 3VO3). All the water molecules in the crystal structure were deleted, bond orders were assigned, hydrogens were added and the protein was then further refined for the docking studies by processing it using Schrodinger's Protein preparation wizard. This procedure minimizes the protein to 0.30 Å RMSD using OPLS-2005 force field. Ligands were

prepared using build panel in maestro. Further the ligands were prepared for docking using LigPrep tool and were energy minimized using MMFF Force Field. Glide Grid generation panel has been used to generate receptor grid for docking. Default SP (Standard Precision) docking protocol was used to dock the library ligands.

Molecular docking studies were performed using the GLIDE program (Masetro version 10.1, Schrodinger, LLC, New York, 2015) to understand the interaction of ligands with 3VO3. The Maestro user interface (version 10.1, Schrodinger, LLC, New York, 2015) was used to set up and execute the docking protocol and also for analysis of the docking results. Validation of docking protocol was done by redocking. Human VEGFR2 (PDB ID: 3VO3 protein) was selected for docking studies and was prepared for docking through protein preparation wizard, energy minimization has been carried out using OPLS2001 force field. Structures of the ligands were sketched using built panel on maestro and prepared for docking through Ligprep module (energy minimized using MMFF force field). GLIDE grid generation wizard has been used to define the docking space.

5.3 EXPERIMENTAL PROCEDURES

5.3.1 SYNTHESIS OF PYRAZOLO BENZPYRIDAZINE DERIVATIVES[12]

STEP1: Synthesis of Ethyl 3 –oxo-2-(2-substituted phenylhydrazinylidene)butanoate:

Various substituted anilines (0.39mol) were dissolved in a mixture of concentrated Hydrochloric acid (15ml) and water (15ml) and cooled to 0-5°C in ice bath, then it was added to a cold saturated solution of sodium nitrite (0.58mol) with constant stirring. The diazonium salt thus formed was filtered into a cooled solution of ethyl acetoacetate (0.39mol) in ethanol and sodium acetate in water (to make it alkaline). The solid was collected and recrystallized from methanol.

STEP2: Synthesis of 3-Acetylbenz pyridazine-4(1*H*)-one derivatives:

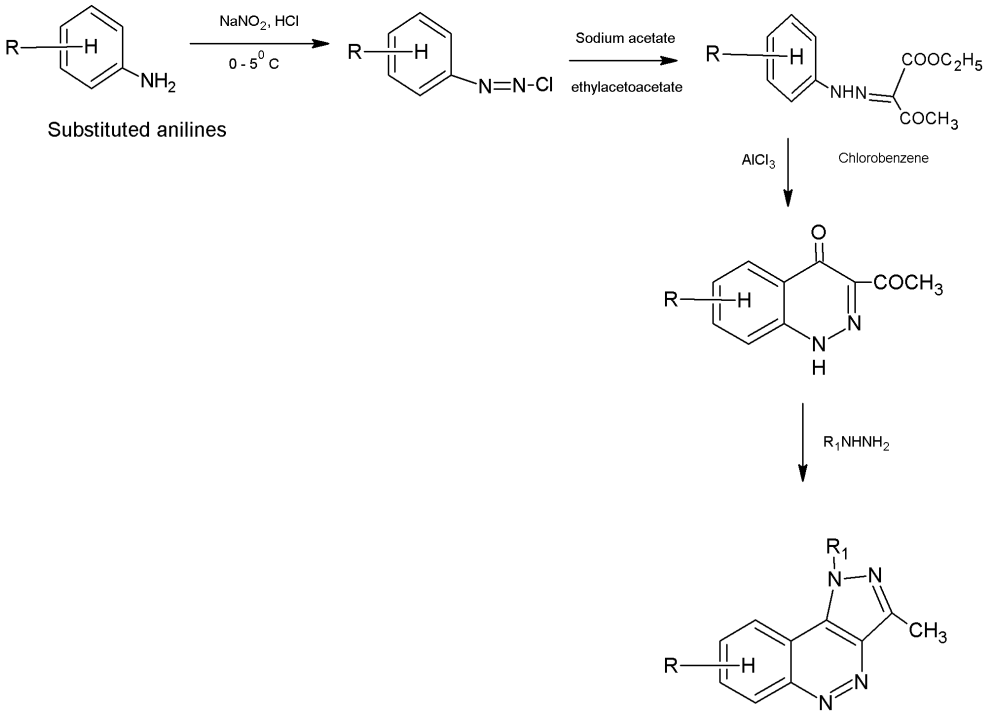
To Ethyl 3 –oxo-2-(2-phenylhydrazinylidene)butanoate(0.01mol) was added anhydrous Aluminium chloride (0.02 mol). Chlorobenzene (30ml) was added in order to dissolve the solids and the mixture was then refluxed for 1hr.the complex formed was decomposed with concentrated hydrochloric acid(30 mL) and diluted with cold

water.The product was filtered, washed with water, dried and recrystallized from methanol.

STEP3: Synthesis of Pyrazolo benzpyridazine derivatives:

A mixture of Ethyl 3 –oxo-2-(2-phenylhydrazinylidene)butanoate(0.005mol) and hydrazine hydrate/phenyl hydrazine (0.02mol) in ethanol was refluxed for 3 hrs. The product formed was collected and recrystallized from ethanol. Melting point and percentage yield of the above compound are determined.The scheme of synthesis is given in scheme.

SCHEME OF SYNTHESIS OF PYRAZOLO BENZPYRIDAZINE



Pyrazolo benzpyridazine derivatives

Table 2: Various substitutions for the synthesised compound

COMPOUND CODE	Substitution Position	
	R	**R_1**
Pz-1	Cl	H
Pz-2	Cl	C_6H_5
Pz-3	F	C_6H_5
Pz-4	NO_2	H
Pz-5	SO_2NH_2	H
Pz-6	SO_2NH_2	C_6H_5

5.3.2 PHYSICOCHEMICAL STUDIES AND CHARACTERIZATION OF SYNTHESIZED COMPOUNDS

5.3.2.1 Melting point analysis:

The melting point of the compounds were determined in one end fused capillary tubes on a THERMONIC MODEL-C-LMP-1,(Campbell melting point) apparatus was used to evaluate the purity of the synthesized compounds and were uncorrected.

5.3.2.2 Thin layer chromatography (TLC):

All the reactions were monitored using TLC. Purity of the synthesized compounds were assessed by a single spot in thin layer chromatography. The spot were visualised using Iodine chamber. The mobile phase used was chloroform: methanol - 0.2: 9.8.

Determination of R_f value is an important technique to identify the formation of synthesized compounds and to determine the purity of the compound. R_f value is the characteristic for each of the compound.

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

5.3.3 SPECTRAL STUDIES:

The structures of the compounds were characterised by Infrared Spectroscopy, Proton and ^{13}C Nuclear Magnetic Resonance and Mass Spectroscopy analysis.

5.3.3.1 Infrared spectral analysis (IR):

The infrared spectral study was done on JASCO FTIR 4100 using KBr disc.The spectral data is expressed in wave numbers (cm^{-1}).

5.3.3.2 Nuclear Magnetic Resonance spectral analysis (^{1}H NMR & ^{13}C NMR)

The NMR spectra of the synthesized compounds were recorded by Bruker Fourier, Transform-NMR using TMS (Tetramethylsilane) as internal standard. The PMR (Proton Magnetic Resonance) and Carbon-13 Magnetic Resonancespectroscopic values were measured in δ ppm in DMSO-d_6.

5.3.3.3 Mass Spectral analysis:

The mass spectra of newly synthesized compounds were recorded on Shimadzu LCMS-2010 EV instrument. The mass of the compounds are expressed in m/z values.

5.4. *IN VITRO* STUDIES

5.4.1 *In vitro* Antioxidant study

5.4.1.1 DPPH (α, α- diphenyl -β- picryl hydrazyl) Free Radical Scavenging Assay

Principle

The molecule of 1, 1-diphenyl-2-picrylhydrazyl (α, α- diphenyl -β- picryl hydrazyl; DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour, characterized by an absorption band in ethanol/methanol solution centred at about 520 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present). Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is

$$Z\bullet + AH = ZH + A\bullet.$$

Procedure[59]

The antioxidant activity of the compound was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH radical (Blois method).0.3mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 1ml of various concentrations of sample and the reference compound (10, 20, 30, 40 and 50 µg/ml), were shaken vigorously and left to stand in the dark at room temperature for 30 min and then absorbance was measured at 517 nm against a blank. Reference compound used here was ascorbic acid. A control reaction was carried out without the test sample. All the tests were performed in triplicate in order to get the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Antiradical activity was expressed as percentage inhibition (I %) and calculated using the following equation:

$$\text{Percentage inhibition (I \%)} = \frac{\text{(Abs of control- Abs of sample)}}{\text{(Abs of control)}} \times 100$$

Different sample concentrations were used in order to obtain calibration curves and to calculate the IC_{50} values. (IC_{50} - concentration required to obtain a 50% radical scavenging activity).

5.4.1.2 ABTS [2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid] Assay

Principle

ABTS decolourization assay is an inhibition method. The peroxidase substrate 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), forms a relatively stable radical (ABTS+) upon one electron oxidation. This assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical and inhibits the absorption of the radical cation which has characteristic long-wavelength absorption spectrum showing maxima at 660, 734, and 820nm.The relatively stable ABTS radical has a green colour and is quantified spectrometrically at 734nm.

Procedure[60]

ABTS radical scavenging activity of the compound was measured by Rice-Evans method. ABTS was dissolved in water to a 7mM concentration. ABTS radical cation (ABTS+) was produced by reacting ABTS stock solution with 2.45mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12- 16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the study, ABTS solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.70 (± 0.02) at 734nm and equilibrated at 30^{o}C. After addition of 1ml of diluted ABTS solution to various concentrations of sample or reference compound (ascorbic acid), the reaction mixture was incubated for 6min and then absorbance was measured at 734 nm against a blank. A control reaction was carried out without the sample. All the tests were performed in triplicate in order to get the mean values. The percentage inhibition of ABTS by the sample was calculated according to the formula:

$$\text{Percentage inhibition (I \%)} = \frac{\text{(Abs of control- Abs of sample)}}{\text{(Abs of control)}} \times 100$$

Different sample concentrations were used in order to obtain calibration curves and to calculate the IC_{50} values. (IC_{50} - concentration required to obtain a 50% radical scavenging activity).

5.5 *IN VITRO* CYTOTOXICITY ASSAY (MTT ASSAY)[61,62]

Cell line used

The human colon cancer cell line (HT-29) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% foetal bovine serum (FBS). The cells were maintained at 37^{0}C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

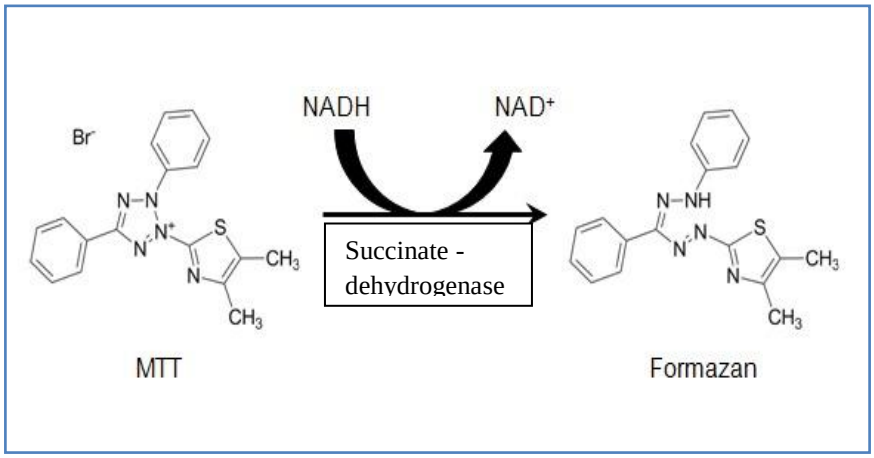
The monolayer cells were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of

$1x10^5$ cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^0C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37^0C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT assay

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore,the amount of formazan produced is directly proportional to the number of viable cells.

Structures of MTT and colored formazan product.



After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO

and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

$$\% \text{ Cell Inhibition} = 100 - \frac{(\text{Abs of sample})}{(\text{Abs of control})} \times 100$$

Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and IC_{50} was determined using GraphPad Prism software.

5.6 *IN VIVO* ANIMAL STUDY

TOXICOLOGICAL EVALUATION OF SYNTHESISED COMPOUND

1. ACUTE ORAL TOXICITY STUDY

Acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a substance or multiple doses given within 24 hours to the animals.

Table 3: Test substance details

Name	Pyrazolo benzpyridazine(Pz-5)
Colour	Yellow colour
Nature	Dry powder

Table 4: Experimental protocol

Name of the study	**Acute toxicity**
Guideline followed	OECD 423 method – Acute toxic class method.
Animals	Healthy young adult albino Wistar rat, nulliparous, non –pregnant.
Body weight & sex	150-200 g & Female
Administration of dose	5,50,300,2000mg/kg
Route of administration	Oral by using rat oral feeding needle

Vehicle	Carboxy methyl cellulose (CMC)
Room temperature	22ºc±3ºc
Humidity	45-55%
Light	12h:12h (light:darkcycle)
Feed	Standard laboratory animal food pellets with water ad libitum

After administration of the drug, food is withheld for further 1-2 hours. The time of death if any, is recorded.

Table 5: Study period and observation parameters

Initial observation	First 30 minutes and periodically 24 hr
Special attention	First 1-4 hr after drug administration
Long term observation	Upto 14 days
Direct observation parameters	Tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.
Additional observation parameters	Skin and fur, eye and mucous membrane, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour patterns

Study procedure.

Acute oral toxicity was performed as per Organization for Economic co-operation for development (OECD) guideline 423 methods. Following the period of fasting, animals were weighed and test substance was administered orally through gavage using specially designed rat oral needle. After the administration of test substance, food was withheld for 2 hours but not water. Animals are observed individually after at least once during the first 30 minutes, periodically during the first 24hrs, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days. Those animals which are found dead or in extreme distress if any are removed and humanely killed for animal welfare reasons.[63]

5.7 EVALUATION OF ANTICANCER ACTIVITY OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR(VEGFR) INHIBITORS ON DMH INDUCED COLON CARCINOMA

5.7.1 Selection of animals for the study

Table 6: Selection of animals for pharmacological evaluation

Species	Sprague Dawley
Age	50 days
Body weight	80-100 gm
Gender	Female

ANIMALS AND MANAGEMENT

Female Sprague-dawley rats 50 days of age, and 80-100 g body weight were offered by KMCH College of Pharmacy, Coimbatore. All the rats were kept at room temperature and allowed to acclimate in standard conditions under 12 hours light/ 12 hours dark cycle in the animal house. Animals are fed with commercial pellet diet and water ad libitum freely throughout the study. The experimental procedure was approved by IAEC (Institution of Animal Ethical Committee) of KMCH governed by CPCSEA, Government of India. Proposal number: KMCRET/M.Pharm/05/2014-15.

5.7.2 Experimental design

Table 7:Experimental design for the study

Group	No of animals	Group Specification
Group I	6	Normal Control
Group II	6	Only DMH(20 mg/kg)
Group III	6	DMH+ Standard (5-FU,20 mg/kg)
Group IV	6	DMH+ Compound PZ-5 (Low dose-100 mg/kg)
Group V	6	DMH+ Compound PZ-5 (High dose- 200 mg/kg)

5.7.3 Induction of Colon cancer using DMH[64]

PRINCIPLE

- 1,2-Dimethyl hydrazine(DMH), a potent colon specific carcinogen and an alkylating agent can cause colon cancer in a reproducible in vivo experimental system for studying non-familial forms of colon carcinoma.
- In which, DMH will be converted to diazonium ions,azoxymethane(AOM) and methylazoxymethanol(MAM) in presence of NAD^+ -dependent dehydrogenase enzyme.These intermediates alkylate colonic mucosal DNA and results in oxidative stress followed by delayed repair of damaged DNA leads to the accumulation of multiple mutations such as Apc,K-ras,b-catenin and thus leads to susceptibility of specific adenocarcinoma of colon.
- Experimental colon cancer induced by DMH in rats mimics human colon cancer and is therefore, an ideal model for chemoprevention studies.

Requirements

Table 8: Requirements for induction of cancer

Carcinogen	1,2-Dimethyl hydrazine
Dose	20 mg/kg
Solvent	0.9 % sodium chloride
Alkalinisation	Sodium hydroxide
Cleaning solution	Sodium carbonate

a. Preparation and induction of DMH solution:

The DMH was purchased from sigma chemicals, Mumbai, India and was stored according to the manufacturer label (2-8^0 C) to prevent its decomposition. The DMH solution was freshly prepared at 20 mg/kg. The DMH was dissolved in 1 mMol/L EDTA-normal saline; the pH was adjusted to 6.5 with 1 Mol/L NaOH to ensure the pH suitability and the stability of the chemical. The resultant solution of the carcinogen was used immediately after preparation. DMH was injected subcutaneously (s.c.) at a dose of 20mg/kg/body weight once a week for ten consecutive weeks on the dorsal back of the animal.

b. Clean up following injection:

After DMH induction the excess amount of DMH and the prepared area were cleaned and chemically inactivated by using a dilute solution of sodium carbonate. Materials used during carcinogen administration were disposed by incineration in compliance with institution biosafety guidelines.

c. Preparation of 5-Fluorouracil solution.

20mg/kg was dissolved in 10 ml of 1 % CMC as vehicle was freshly prepared daily and given via orally to the standard group.

d. **Preparation of sample:**

100 mg /kg and 200 mg/kg of test compound Pz-5 were dissolved in 1% CMC and it was prepared freshly and given via oral route to group IV& V respectively.

5.7.4 Determination of body weight [64]

All the rats were weighed for every 7 days after tumour inoculation. Average gain in body weight was determined and recorded.

5.7.5 Estimation of Tumerogenesis

5.7.5.1 Estimation of Tumour Burden [64]

Number of tumours formed in each animal is called tumour burden. It is also find out by dissection of animals on final day of study.

5.7.5.2 Estimation of Tumour Weight

Tumour weight was estimated according to the method of Geren et al. The resultant solid tumor was considered to be prelate ellipsoid with one long axis and two short axis. The tumour weight was calculated by measuring the weight of colon (approx.10cm).

5.7.6 Estimation of hematological parameters

Blood collection

After the end of the treatment period (ie. 30days) the animals were anaesthesised with ketamine (i.p route) and its blood was collected by retro orbital puncture with addition of EDTA for the enumeration of blood cells ie, RBC, WBC and estimation of haemoglobin. The estimation of various biochemical parameters carried out by using blood sample without adding EDTA.

Separation of serum

For the estimation of the biochemical parameters such as Alkaline phosphatase (ALP), Serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), Serum creatinine and total protein. The serum was separated from the blood by centrifuging at 10,000 rpm for 10 minutes. The separated serum was collected and used for the estimation of parameters.

5.7.6.1 Enumeration of red blood cells (RBC) [65]

Requirements

- RBC diluting fluid (Hayem's fluid)
- Counting chamber (Neubauer's chamber)
- RBC pipette
- Microscope with 45 X lens

Procedure

The RBC pipette was filled with blood upto the mark 0.5. RBC diluting fluid (Hayem's fluid) was filled upto the mark 101. The pipette was rolled between the palms to ensure thorough mixing of blood with diluting fluid and kept for sometime. The counting chamber was placed and the RBC squares were focussed under low power and after identification of the markings turn to high power. The first 3-4 drops of blood mixture was discarded and it was mixed once again. The counting chamber was charged with the mixed blood. After that mount the slide and allowed the fluid to settle down by using a 45 X lens. The RBC was counted in the corner and middle squares. The number of cells were expressed as 10^6cells/mm^3.

5.7.6.2 Enumeration of white blood cells (WBC) [65]

Requirements

- WBC diluting fluid (Turk's fluid)
- Counting chamber (Neubauer's chamber)
- WBC pipette
- Microscope with 45 X lens

Procedure

The WBC pipette was filled with blood upto the mark 0.5. WBC diluting fluid (Turk's fluid) was filled upto the mark 11. The pipette was rolled between the palms to ensure thorough mixing of blood with diluting fluid and kept for sometime. The counting chamber was placed and the WBC squares were focussed under low power and after identification of the markings turn to high power. The first 3-4 drops of blood mixture was discarded and it was mixed once again. The counting chamber was charged with the mixed blood. After that mount the slide and allowed the fluid to settle down by using a 45 X lens. The WBC was counted in the corner and middle squares. The number of cells was expressed as 10^3 cells/mm^3

5.7.6.3 Estimation of haemoglobin [65]

Principle

This method is based on the conversion of heamogobin to acid haematin by treatment with 0.1 N hydrochloric acid. The brown colour formed due to acid heamatin was matched against a glass standard on a comparator.

Requirements

- Sahil's hemometer
- Hb pipette
- 0.1 N HCl

Procedure

The heamoglobinometer tube was filled with 0.1 N HCl up to the marking 10. To this 20 µL of blood was added by using pipette. The superfacial acid was and rinsed

repeatedly till all the blood in the pipette washed out in the acid. Mixed thoroughly by stirrer and allowed to stand for 10 minutes. A clear brown colour solution was formed due to the formation of acid haematin. Dilute the solution by adding drop by drop of distilled water. The colour of the diluted solution was compared with that of standard. Reading was noted directly from the graduated tube as g/ 100 mL or as percentage of haemoglobin.

5.7.7 Estimation of serum biochemical parameters[66,67]

5.7.7.1 Estimation of alkaline phosphatase (ALP)

Method

Kinetic photometric test-according to IFCC (International Federation of Clinical Chemistry and Laboratory Medicine)

Principle

ALP hydrolyses P-Nitrophenylphosphate to P-Nitrophenol and phosphate.

Reaction

P-Nitrophenyl phosphate + H_2O ⟶ P-Nitrophenol + phosphate

Reagents

Table 9: Composition of ALP reagent

R_1	Diethanolamine	1.5Mol/L
	Magnesium chloride	0.6m Mol/L
R_2	p-Nitrophenyl Phosphate Analogue	0.070mMol/L
	Sodium azide 10 %	

Procedure

a. Mixed 0.8 mL of reagent-1 with 0.2mL of reagent-2 in a 2mL eppendroff tube.

b. To this added 25µL of serum.

c. Mixed well and run the sample

5.7.7.2 Serum glutamate oxaloacetate transaminase(SGOT)

Principle

$$\text{L-aspartate + 2-oxaloglutarate} \xleftrightarrow{\text{SGOT}} \text{L-Glutamate + oxaloacetate.}$$

$$\text{Oxaloacetate + NADH + } H^{+} \xleftrightarrow{\text{LDH}} \text{D- Malate + } NAD^{+}$$

This modified formula for the assay of AST/SGOT, as recommended by the IFCC (International federation of clinical chemistry). The IFCC reference method includes pyridoxal phosphate (PP) function as coenzyme in AA transfer, therefore addition of PP results in increased enzyme activity. It avoids falsely low activity in samples containing insufficient endogenous PP, e.g. from patients with myocardial infarction, liver disease and intensive care patients.

Method

Optimized UV test according to the IFCC (International Federation of Clinical Chemistry and laboratory medicine)

Table 10: Composition of SGOT reagent

R1	TRIS pH7.8	80mMol/L
	Preservatives.	
	Stabilizers.	
R2	L-Aspartate	240mMol/L
	NADH	0.18 mMol/L
	Pyridoxal-5-phosphate	
	Lactate dehydrogenase	≥900U/L
	Malate dehydrogenase	≥600U/L
	Alpha-ketoglutarate.	12mmol/L

Assay procedure

a. Mixed 800 µL of reagent-1 with 200 µL of reagent-2 in a 5 mL test tube

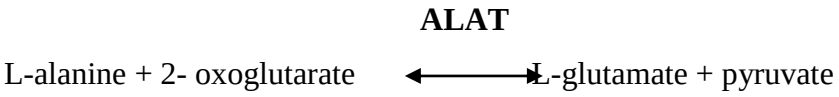
b. To this added 100 µL of serum.

c. Mixed well and run the sample

d. The readings were taken in a semi autoanalyser(Model photometer 5010)

5.7.7.3 Serum glutamate pyruvate transaminase (SGPT)

Principle



Addition of pyridoxal -5-phosphate (P-5-P) stabilizes the transaminases and avoid falsely low values in samples containing insufficient endogenous P-5-P, e.g from patient with myocardial infarction, liver disease and intensive care patients.

Method

Kinetic UV test. According to international federation of clinical chemistry and laboratory medicine (IFCC)

Reagents

Table 11: Composition of SGPT reagent

R1	L- alanine	≥200mMol/L
	LDH (lactate dehydrogenase)	≥1500 U/L
	2-Oxoglutarate	15mMol/L
R2	NADH	1.05mMol/L
	Pyridoxal -5-phosphate	
	Alpha-ketoglutarate	>35mMol/L

Procedure

a. Mixed 800 µL of reagent-1 with 200 µL of reagent-2 in a 5ml test tube

b. To this added 100 µL of serum.

c. Mixed well and run the sample.
d. The readings were taken in a semi autoanalyser (Model photometer 5010)

5.7.7.4 Estimation of Creatinine

The amount of creatinine present was estimated according to Jaffe method (modified)

Principle

In an alkaline medium, picric acid reacts with creatinine which forms an orange coloured complex with the alkaline picrate. Intensity of colour formed is measured which is directly proportional to the amount of creatinine present in the sample.

Reaction

Creatinine + Alkaline picrate $\longrightarrow$ orange coloured complex

Requirements

Reagent 1: Standard creatinine

Reagent 2: Picric acid solution

Reagent 3: Sodium hydroxide solution

Procedure

To 500 µL of reagent 2 and 500 µL of reagent 3 were taken in a 5 mL test tube and mixed. To this reagent about 100 µL of serum was added and mixed well. The readings were taken immediately in a semi autoanalyser (Model photometer 5010).

5.7.7.5 Estimation of Total protein

The amount of protein was determined by biuret method.

Principle

Protein forms coloured complexes with cupric ions in alkaline medium

Reagents

Reagent 1

Cupric sulphate – 6 mmol/L

Potassium iodide- 15 mmol/L

Reagent 2

Protein (standard)- 6g/100 mL

Procedure

To 1 mL of reagent 1 in a 5 mL test tube was added 20 µL of serum and mixed well and incubated at a room temperature for 15 minutes and read the absorbance.

5.7.8 *IN VIVO* antioxidant activity

Requirements

Saline

Disodium hydrogen phosphate

Potassium dihydrogen phosphate

Sodium chloride

Preparation of 0.1M Phosphate buffer saline buffer(pH– 7.4)

2.38g Disodium hydrogen phosphate,0.19g potassium dihydrogen phosphate and 8g sodium chloride were weighed and dissolved in distilled water and the volume was made up to 1000ml.

Preparation of homogenate

After blood samples were withdrawn, SD rats were sacrificed by an excessive dose of anesthetic ether and colons were dissected out and perfused with an ice cold saline transcardially. From this approx.1.5gms of colon was weighed. To this 20 ml of the buffer (0.1M PBS) was added. It was homogenized with motor driven Teflon coated homogeniser (RQ-127A, REMI MOTORS group, Mumbai,Maharashtra, India) in an ice cold condition. It was centrifuged at 1000rpm for 10 minutes at 4 ºC. The supernatant was collected and used for the *in vivo* antioxidant activity estimation.The homogenate was used for the following estimations, namely, GSH, catalase, SOD, GPx, Lipid peroxidation.

5.7.8.1 Estimation of Catalase[67]

The activity of catalase was assayed by the method of Beers and Sizer et al, 1952.

Requirements

Dichromate Acetic acid Reagent

0.01M Phosphate Buffer (pH-7.0)

0.2M Hydrogen peroxide.

Principle

The normal antioxidant activity of the enzyme catalase is due to acceleration of decomposition of hydrogen peroxide to water and oxygen. This method is based on the principle of measuring the rates of decomposition of hydrogen peroxide by the enzyme catalase by measured spectrophotometrically at 570 nm, since hydrogen peroxide has the absorbance at this range.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Procedure

To one ml of tissue homogenate, 4 ml of hydrogen peroxide and 5 ml of phosphate buffer was added and mixed. From this one ml of solution was taken and mixed with Dichromate acetic acid reagent and allowed to incubate for 30 minutes at room temperature. The absorbance was measured at 570 nm. The activity of catalase was expressed as µmole of H_2O_2 consumed/min/mg protein.

5.7.8.2 Estimation of superoxide dismutase (SOD)

The activity of the superoxide dismutase was assayed by the method of Fridvich et.al.[67]

Requirements

Adrenaline

Carbonate Buffer (pH-10.2)

Principle

Pyrogallol autoxidizes rapidly in aqueous solution, where the reaction will be faster at higher pH, and leads to the formation of several intermediate products. Thus the solution first becomes yellow-brown with a spectrum between 400 and 425nm. Molecular oxygen, carrying two unpaired electrons with parallel spins, has a preference for univalent reduction because spin restrictions arise when reduction with electron pairs is attempted. The recently discovered enzyme superoxide dismutase rapidly dismutases univalently reduced oxygen $O_2^{\cdot-}$ i.e., the superoxide anion radical ($2O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2$). The enzyme has proven to be a useful probe for studying the participation of the radical in reactions involving oxygen such as autooxidations. Thus $O_2^{\cdot-}$ has been shown to be involved in the auto-oxidation of sulphite, adrenalin and 6-hydroxydopamine.

Procedure

5% homogenate was mixed with 75 Mm Tris-HCL (pH 8.2), 30 mM EDTA, and 2 mM pyrogallol respectively. Then, the absorbance was measured at 420 nm. The percentage of inhibition was calculated depending on that the ability of enzyme to inhibit oxidation.The antioxidant activity of SOD enzymes was expressed as units/min/mg protein.

5.7.8.3 Estimation of glutathione peroxidase (GPx)

The activity of the Glutathione peroxidise was assayed by the method of Paglia and valentine et.al. 1967.

Requirements

0.32 M Phosphate buffer, pH 7.0

0.8Mm EDTA

10 Mm sodium azide

3 Mm reduced glutathione

2.5mM Reduced H_2O_2

10 percentage TCA

0.3 M Disodium hydrogen phosphate

DTNB Solution (40 mg of DTNB in 100 ml of 1% sodium citrate)

Reduced glutathione

Principle

This assay is based on the principle reduction of hydrogen peroxide (H_2O_2) by gluthathione peroxidise through simultaneous oxidizing of reduced glutathione (GSH) to form oxidized glutathione (GSSG).GSSG is further then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP+ which resulting in decreased absorbance at 340 nm and recycling the GSH. The decrease in absorbance at 340 nm is directly proportional to the GPx concentration.

Procedure

To 0.1 ml of the tissue homogenate, 0.2 ml of EDTA, Sodium azide, hydrogen peroxide were added and mixed. Then 0.4 ml of phosphate buffer was added and allowed to incubate at room temperature. The reaction was arrested by the addition of 0.5 ml of TCA .The reaction mixture was centrifuged at 2000 rpm and supernatant was collected. To 0.5 ml of the supernatant 4 ml of disodium hydrogen phosphate and 0.5 ml of DTNB were added and the colour developed was read immediately at 420 nm .The activity of glutathione peroxidise was expressed as µ moles of glutathione oxidized/min/mg.

5.7.8.4 Estimation of reduced glutathione (GSH)

The activity of reduced glutathione was assayed by the method of Anderson (1985).

Requirements

10% TCA

0.6 mM 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate

0.2 M Phosphate buffer, pH 8.0

Principle

DTNB is a disulfide compound, which was reduced by sulphadryl groups present in GSH. This reduction leads to the formation of yellow colour, which was measured at 412 nm.

Procedure

To 1 ml of the homogenate, 1 ml of the TCA solution was added and centrifuged. The supernatant was collected and the precipitate formed was removed. To 0.5 ml of supernatant 2 ml of DTNB was added, the volume was made up to 3 ml with phosphate buffer. Then absorbance was read at 412 nm .The amount of glutathione was expressed as µ/mg protein.

5.7.8.5 Determination of lipid peroxidation

Lipid peroxidation was measured by the modified method of Ohkawa et.al.[67]

Requirements

Thiobarbituric acid 0.37 $^{0}/_{0}$

0.25 N HCl

15 $^{0}/_{0}$ TCA

Principle

This assay is based on the reduction between thiobarbituric acid with malonyldialdehyde which is a formed as a result of polyunsaturated fatty acid oxidation.This reaction leads to the formation of pink colored TBA-MDA complex which is measured at 532 nm.

Procedure

To 0.1 ml of sample, 2 ml of TBA-TCA-HCl reagent (ratio of 1:1) was added mixed and kept in a water bath for 15 minutes. Afterwards the solution was cooled and supernatant was removed and absorbance was measured at 535 nm against reference blank. The level of lipid peroxides was given as nm moles of MDA formed/mg protein.

5.7.8.6 Estimation of proteins

Protein was estimated by the method of Lowry *et.al* [67]

Requirements

Alkaline copper reagent

Solution A: 2 $^{0}/_{0}$ sodium carbonate in 0.1 N NaoH.

Solution B: 0.5 $^{0}/_{0}$ copper sulphate in 1 $^{o}/_{o}$ sodium potassium tartarate 50 ml of solution A was mixed with 1 ml of solution B just before use.
Folin's phenol reagent (commercial reagent, 1:2 dilutions),

Bovine serum albumin (BSA).

Principle

This method is a combination of both Folin-ciocalteau and biuret reaction which involves two steps

Step: 1

Protein binds with copper in alkaline medium and reduces it to Cu^{++}.

Step: 2

The Cu++ formed catalyses the oxidation reaction of aromatic amino acid by reducing phosphomolybdotungstate to heteropolymolybdanum ,which leads to the formation of blue colour and absorbance was measured at 640 nm.

Procedure

To 0.1 ml of the homogenate, 0.9 ml of water, 4.5 ml of alkaline copper sulphate reagent were added and allowed to stand in the room temperature for 10 minutes. To this 0.5 ml of Folin's reagent was added. After 20 minutes, the colour developed was measured at 640 nm. The level of protein present was expressed as mg/g/ tissue or mg/dl.[91]

5.7.9 Estimation of mitochondrial TCA cycle enzymes activity

Isolation of mitochondria

The isolation of mitochondria and microsomes was carried according to the method of Johnson and Lardy et.al (1967) and Hanioka et.al (1997) respectively.

Requirements

0.05 M Tris –HCl buffer, pH 7.4 containing 0.25 M sucrose.

0.05 M Tris – HCl buffer, pH 7.4 containing 0.15 M potassium chloride.

Preparation of homogenate (20 $^{0}/_{0}$)

The isolated kidney and liver was washed with saline solution. From this 2 gms of kidney and liver was weighed. To this 20 ml of the buffer (0.05 M Tris –HCl containing 0.25 M sucrose) was added. It was homogenized with motor driven Teflon coated homogenizer in an ice cold condition. It was centrifuged at 1000 rpm for 10 minutes at 4 ^{0} C. The supernatant was collected again it was centrifuged at 600 x g for 10 minutes .The supernatant was collected and centrifuged again at 1500 x g for 5 minutes, which results in the formation of the mitochondrial pellet. The pellet formed was washed and suspended in the same buffer. The suspended pellet was centrifuged again at 105,000 x g for 60 minutes, the supernatant solution was decanted and the resultant pellet was again re suspended in the 0.05 M Tris buffer containing 0.15 M KCl.

5.7.9.1 Assay of succinate dehydrogenase

The enzyme activity was assayed according to the method of Slater and Bonner (1952).

Requirements

0.3 M Phosphate buffer, pH 7.6

0.03 M EDTA

0.03 M Potassium cyanide

0.4 M Succinate

3 % Bovine serum albumin

0.075 M Potassium ferricyanide

Procedure

To 1 ml of phosphate buffer, 0.1 ml of EDTA, 0.1 ml of bovine serum albumin, 0.3 ml of sodium succinate ,0.2 ml of potassium ferricyanide were added mixed and the final volume was made up to 2.8 ml with water. To this reaction mixture 0.2 ml of mitochondrial suspension was added immediately before measuring the absorbance allowed .The change in absorbance was recorded for 5 minutes with 15 seconds interval at 420 nm. The succinate dehydrogenase activity was measured as µ moles of succinate oxidized/min/mg protein.[92]

5.7.10 Histopathology of Colon Tumour

Histopathology is the microscopical study of tissues for pathological alterations. This involves collection of morbid tissues from biopsy or necropsy, fixation, preparation of sections, staining and microscopical examination.

Collection of materials

Thin pieces of 3 to 5 mm thickness were collected from tissues showing gross morbid changes along with normal tissue.

Fixation

- Kept the tissue in fixative for 24 – 48 hr at room temperature.
- The fixation was useful in the following ways:
- Serves to harden the tissue by coagulating the cell protein.
- Prevents autolysis
- Preserves the structure of the tissue
- Prevents shrinkage
- Common fixative: 10%Formalin

5.7.10.1 Haematoxylin and eosin method of staining

Deparaffinised the colonic section by xylol 5 to 10 minutes and removed xylol by absolute alcohol. Then cleaned the section in tap water and stained with haematoxylin

for 3-4 minutes and again cleaned under tap water. Allow the sections in tap water for few minutes and counter stained with 0.5 % eosin until section appears light pink (15 to 30 seconds) and then washed in tap water. Blottedand dehydrated in alcohol and cleared with xylol (15 to 30 seconds).Mounted on a Canada balsam or DPX mountant and kept the slide dry and remove air bubbles.

5.7.10.2 Immunohistochemistry by COX 2 Expression Study

The pretreated paraffinised colon sections were kept in poly –L-lysine coated slides and these section slides were placed into antigen retrival pretreatment.After the pretreatment, the slides were placed in to the solution jar containing buffer.Covered the pretreated slides with peroxidase blocking agent and incubated for ten minutes.The slides were washed with deionised water and buffer solution periodically.Then covered the section with power blocking solution and incubated it for 10minutes followed by continued washing with buffer solution and 3 changes into it.The pretreated colon sections were covered with primary antibody which are used for process(COX-2) and incubated the sections for 30minutes,again washed with buffer solutions with three changes. Then added secondary antibody and incubated for 30minutes in room temperature. Washed with buffer solutions 3 times and then

placed the slides into substrate DAB solution for ten minutes. Then stained the slides with counter stain haematoxylin solution. Dried and mounted the slides for microscopic observation and placed in to pathologist table. The test results were obtained from microscopically by pathologist.

5.7.12 Statistical analysis

The data were analyzed by using GRAPH PAD PRISM, VERSION 6.05, analysis of variance (ANOVA) followed by Dunnet's and Turkey's multiple comparison of all pairs of columns test was performed.

6. RESULTS AND DISCUSSION

IN SILICO STUDIES

LEAD OPTIMISATION

Lead optimization aims at enhancing the most promising compounds to improve effectiveness, diminish toxicity, or increase absorption. Many of the technologies for lead discovery overlap with lead optimization as researchers attempt to incorporate the best drug characteristics early in the process. While the approaches taken may vary, the central theme is the same: make it better, faster, and more efficient.

Druglikeness is a qualitative concept used in drug design for how "druglike" a substance is with respect to factors like bioavailability. It is estimated from the molecular structure before the substance is even synthesized and tested.

Table 12: Druglikeness Molecular properties (Molinspiration) table (Lipinski Rule Table)

Compound code	Mol. Wt	Log P	No of H Donors	No of H acceptors	No of violations	Mol. Polar Surface Area
Pz-1	218.65	2.594	1	3	0	54.46
Pz-2	294.75	3.94	0	3	0	43.6
Pz-3	278.29	3.42	0	4	0	43.6
Pz-4	305.30	3.22	1	5	0	100.28
Pz-5	263.28	0.61	1	6	0	123
Pz-6	339.38	1.95	0	6	0	112.14

ADME Properties predictions

Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties of drug candidates or environmental chemicals play a key role in drug discovery and environmental hazard assessment**.** All the synthesised benzpyridazine derivatives were subjected to ADME Properties predictions using ADMESAR software, and the predicted Absorption and Distribution properties are given in table: 13 and the predicted metabolic properties are given in table:14.

Table 13: Absorption and Distribution Properties Prediction using ADMESAR

Compound code	Blood-Brain Barrier	Human Intestinal Absorption	Caco-2 Permeability	LogPapp, cm/s	P-glycoprotein Substrate
PZ-1	BBB+	HIA+	Caco2-	1.0776	Non-substrate
PZ-2	BBB+	HIA+	Caco2+	1.4446	Non-substrate
PZ-3	BBB+	HIA+	Caco2+	1.3634	Non-substrate
PZ-4	BBB+	HIA+	Caco2+	1.0872	Non-substrate
PZ-5	BBB+	HIA+	Caco2+	0.9354	Non-substrate
PZ-6	BBB+	HIA+	Caco2-	0.5929	Non-substrate

Caco-2 & $LogP_{app}$ - In vitro model of the human small intestinal mucosa to predict the absorption of orally administered drugs.
P-glycoprotein- ATP- dependent efflux pump with broad substrate specificity.

Table 14: Metabolic properties predictions of synthesised benzpyridazine derivatives

Compound code	CYP450 2D6 Substrate	CYP450 2D6 Inhibitor	CYP450 2C9 Substrate	CYP450 2C9 Inhibitor
PZ-1	Non-substrate	Non-inhibitor	Non-substrate	Non-inhibitor
PZ-2	Non-substrate	Non-inhibitor	Non-substrate	Inhibitor
PZ-3	Non-substrate	Non-inhibitor	Non-substrate	Inhibitor
PZ-4	Non-substrate	Non-inhibitor	Non-substrate	Non-inhibitor
PZ-5	Non-substrate	Non-inhibitor	Non-substrate	Non-inhibitor
PZ-6	Non-substrate	Non-inhibitor	Non-substrate	Non-inhibitor

CYP450 2D6 & CYP450 2C9 - a member of the cytochrome P450 mixed-function oxidase system, involved in the metabolism of xenobiotics in the body.

TOXICITY STUDIES

PROTOX is a webserver for the prediction of oral toxicities of small molecules in rodents. The medial lethal dose (LD50) and the toxicity class is calculated for an input compound based on chemical similarites to toxic compounds. All the synthesised benzpyridazine derivatives were subjected to oral toxicities predictions using PROTOX software, and the compounds were found to be within the applicable range and the predicted toxicity properties are given in table:15.

Table 15 : Oral toxicity properties predictions of synthesised benzpyridazine derivatives

Sl. No	Compound code	Predicted Oral toxicity LD50 value mg/kg	Predicted Toxicity Class	Tox related fragments	Toxicity targets binding[AA2AR, ADRB2, ANDR, AOFA, CRFR1DRD3, ESR1, ESR2, GCR, HRH1, NR1I2, OPRK, OPRM, PDE4D, PGH1, PRGR]	Rat IP LD50 (mg/kg)	Rat IV LD50 (mg/kg)	Rat Oral LD50 (mg/kg)	Rat SC LD50 (mg/kg)
1	PZ-1	1000	4	No toxicity Fragments	No Binding	265,200	110,500	778,000	488,300
2	PZ-2	1680	4	No toxicity Fragments	No Binding	382,100	124,700	1520,000	1158,000
3	PZ-3	1680	4	No toxicity Fragments	No Binding	353,300	180,600	979,200	564,700
4	PZ-4	2200	5	No toxicity Fragments	No Binding	591,400	124,000	547,700	265,400
5	PZ-5	295	3	No toxicity Fragments	No Binding	916,500	881,000	4298,000	1327,000
6	PZ-6	1680	4	No toxicity Fragments	No Binding	979,100	617,900	4484,000	1578,000

AD- applicable domain

Eco-toxicity predictions

GUSAR has been developed according to OECD principles and includes last achievements in the field of QSAR modeling. Quantitative prediction of ecotoxicity for chemical compounds by GUSAR software was utilised for predicting the Ecotoxicity of the designed molecules. The QSAR models were developed for the following endpoints: 96 hr fathead minnow 50% lethal concentration, 48 hr daphnia magna 50% lethal concentration, Tetrahymena pyriformis 50% growth inhibition concentration and Bio concentration Factor. All the synthesised compounds fall in applicable range. All the synthesised benzpyridazine derivatives were subjected to eco-toxicities predictions using **GUSAR** software, and the predicted toxicity properties are given in table:16.

Table 16: Environmental Toxicity predictions report.

Comp. code	Predicted activity						Biodegradation
	Bioaccumulation factor Log10(BCF)	Daphnia magna LC50 - Log10(mol/L)	Fathead Minnow LC50 Log10 mmol/L	Tetrahymena pyriformis IGC50 Log10 (mol/L)	AMES Toxicity	Carcinogens	
PZ-1	1,152	4,986	-1,218	1,392	AMES toxic	Non-carcinogen	Not readily biodegradable
PZ-2	1,716	5,146	-2,082	1,755	AMES toxic	Non-carcinogen	Not readily biodegradable
PZ-3	1,445	5,925	-1,966	1,520	AMES toxic	Non-carcinogen	Not readily biodegradable
PZ-4	0,812	5,119	-1,159	1,223	AMES toxic	Non-carcinogen	Not readily biodegradable
PZ-5	0,467	4,418	-0,244	0,711	Non-AMES toxic	Non-carcinogen	Not readily biodegradable
PZ-6	0,596	5,337	-1,210	0,848	Non-AMES toxic	Non-carcinogen	Not readily biodegradable

Bioaccumulation is the uptake of organic compounds by biota from either water or food.
Daphnia magna-Waterflea

MOLECULAR DOCKING

A library of **54** compounds that contain pyrazolo benzpyridazine derivatives were made, then these compounds were optimized and docked into the active pocket of **VEGFR2** protein (**PDB code: 3VO3**) using the glide program, in order to make a pre-selection of promising compounds.The maestro user interface (version 10.1,Schrodinger, LLC, New York,2015)

was employed to set up and execute the docking protocol and also for analysis of the docking results.According to their docking scores and their interaction with VEGF receptor, the selection of the compounds were made and synthesized. The Docking studies of designed pyrazolo pyridazine compounds with VEGFR(3VO3) are given in the table: 17.

The amino acid interaction studies of the selected compounds were carried out and are given in table: 18. From the docking studies, compound PZ-5 having good glide score and amino acid interaction with Cys919 of 3VO3 as like standard ligand. The docking and aminoacid interactions of selected compounds (PZ-1 to PZ-6) were shown in figure 24-33.

Table 17: Docking studies of designed pyrazolo pyridazine compounds with VEGFR

Designed Lead Code **Bpp**	GLIDE Score	Lipophilic EvdW	PhobEn	PhobEnHB	PhobEnPair HB	HBond
1	-7.25	-5.22	-0.93	0	0	-0.7
2	-6.95	-5.42	-1.18	0	0	0
3	-6.91	-4.91	-1.15	0	0	0
4	-6.8	-5.26	-1.1	0	0	0
5	-6.75	-3.23	-0.53	0	-1.22	-0.93
6	-6.74	- 3.19	-0.53	0	-1.23	-0.94
7	-6.72	-5.35	-0.97	0	0	0
8	-6.64	-3.15	-0.53	0	-1.14	-0.99
9	-6.62	-3.4	0	0	-1.5	-0.93
10	-6.55	-4.44	-1.3	0	0	0
11	-6.53	-4.09	-1.27	0	0	0
12	-6.58	-2.67	0	-1.5	0	-1.32
13	-6.48	-5.01	-0.88	0	0	0
14	-6.38	-3.17	-0.53	0	-0.91	-0.9
15	-6.34	-4.63	-0.95	0	0	0
16	-6.37	-5.08	-0.8	0	0	0
17	-6.3	-3.14	-0.53	0	-0.87	-0.9
18	-6.3	-3.24	-0.53	0	-0.94	-0.75
19	-6.27	-3.14	-0.53	0	-1.06	-0.74
20	-6.22	-2.93	0	0	-1.39	-1
21	-6.21	-4.92	-0.9	0	0	0
22	-6.2	-3.8	0	0	0	-1

23	-6.15	-4.88	-0.9	0	0	0
24	-6.13	-3.49	-0.88	0	0	-0.7
25	-6.13	-4.86	-0.9	0	0	0
26	-6.12	-4.9	-0.85	0	0	0
27	-6.03	-4.78	-0.93	0	0	0
28	-6.37	-4.47	-0.97	0	0	-0.39
29	-7.18	-5.01	-0.85	0	0	-0.89
30	-5.91	-3.14	-0.5	0	-0.62	-0.81
31	-5.8	-3.67	-1.18	0	0	0
32	-5.63	-4.49	-0.75	0	0	0
33	-5.59	-3.61	-0.3	0	0	-0.7
34	-5.47	-3.43	-1.01	0	0	-0.22
35	-5.39	-3.56	-0.78	0	0	-0.07
36	-5.25	-4.33	-0.45	0	0	0
37	-5.1	-3.11	-0.3	0	0	-0.7
38	-5.08	-3.47	-0.28	0	0	-0.35
39	-5.04	-4.77	0	0	0	0
40	-5.03	-3.39	-0.28	0	0	-0.7
41	-5.03	-3.05	-0.53	-0.23	0	-0.45
42	-5	-3.21	-0.45	0	0	-0.66
43	-5.32	-3.42	-0.28	0	0	-0.68
44	-4.74	-4.25	0	0	0	0
45	-4.73	-2.5	-0.13	0	0	-0.69
46	-4.73	-3.76	0	0	0	-0.7
47	-4.67	-3.23	-0.13	0	0	-0.97
48	-5.8	-2.51	0	-1.5	0	-0.83
49	-4.31	-3.72	-0.3	0	0	-0.68
50	-4.29	-3.4	-0.45	0	0	0
51	-4.76	-4	-0.53	0	0	0
52	-4.54	-2.8	-0.33	0	0	-0.7
53	-7.82	-3.41	-0.68	0	-1.22	-1.38
54	-7.28	-4.81	-0.9	0	0	-0.7

Table 18: Amino acid interactions of selected compounds

Molecule Design Code	Comp. Code	GLIDE Dock Score	Amino Acid Interacted	Hydrogen Length	Molecule Linked
Bpp-01	Pz-1	-6.74	Cys919	2.07	Pyrazole
Bpp-19	Pz-2	-6.72	Phe1047	1.00	Benz-Pyridazine
Bpp-30	Pz-3	-6.91	Cys919 Phe1047	2.45	Pyridazine Pyrazole
Bpp-28	Pz-4	-6.75	Cys919	2.39	Pyrazole
Bpp-41	Pz-5	-7.82	Cys919	2.45	Pyrazole
Bpp-38	Pz-6	-7.25	Arg1032 Arg1051 Phe1047	2.00	Pyridazine

Figure 24: 2D Interaction Of N-[3-({2-[(Cyclopropyl Carbonyl) Amino] Imidazo[1,2-B]Pyridazin-6-yl}Oxy)Phenyl]-1,3-Dimethyl- 1H-Pyrazole-5-Carboxamide With 3VO3

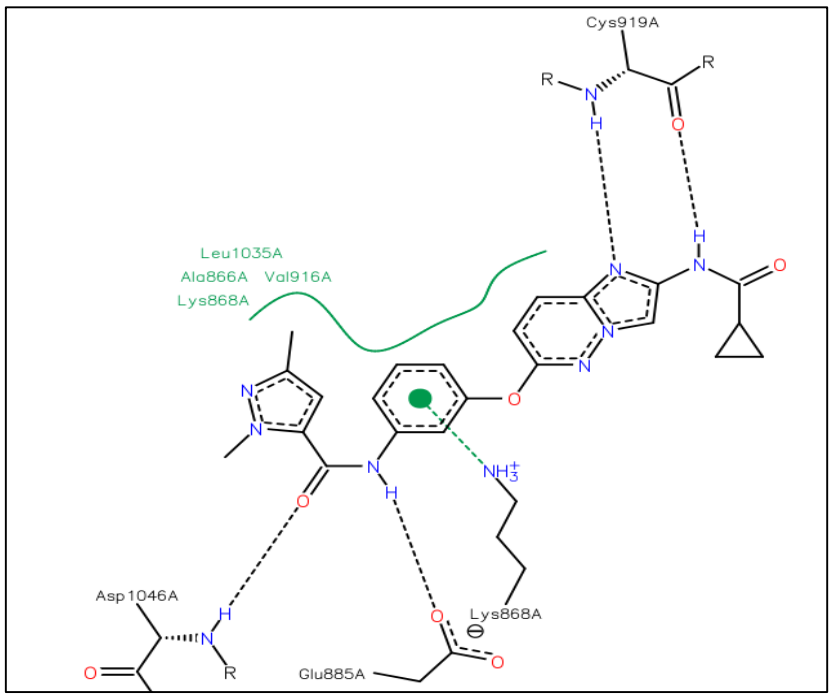


Figure 25: 2 D Interaction of Compound PZ-1

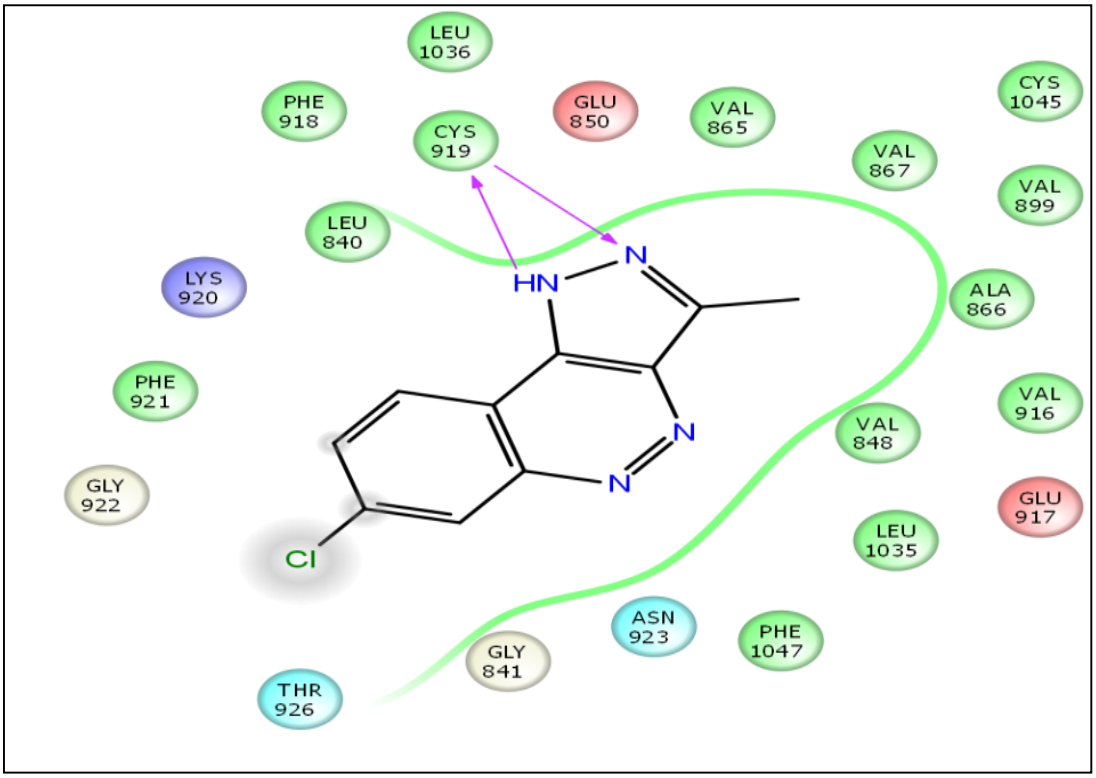


Figure 26: 3 D Interaction of Compound PZ-1

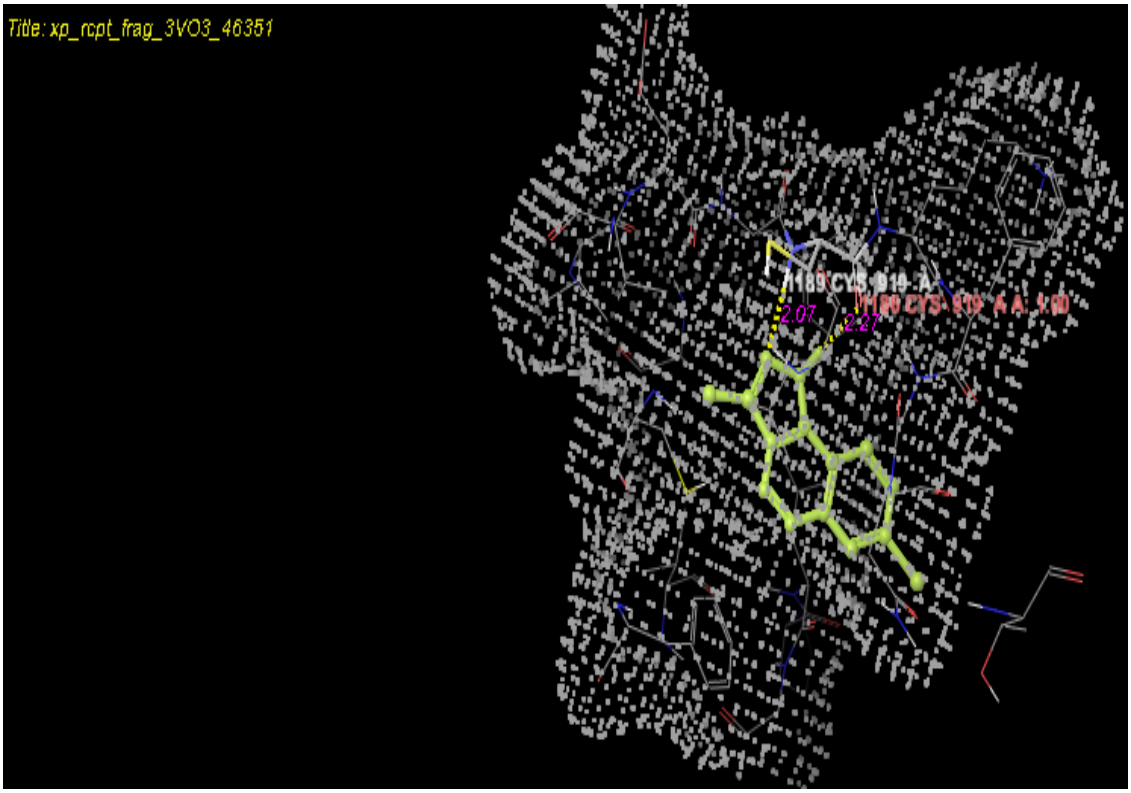


Figure 27: 2 D Interaction Of Compound PZ-2

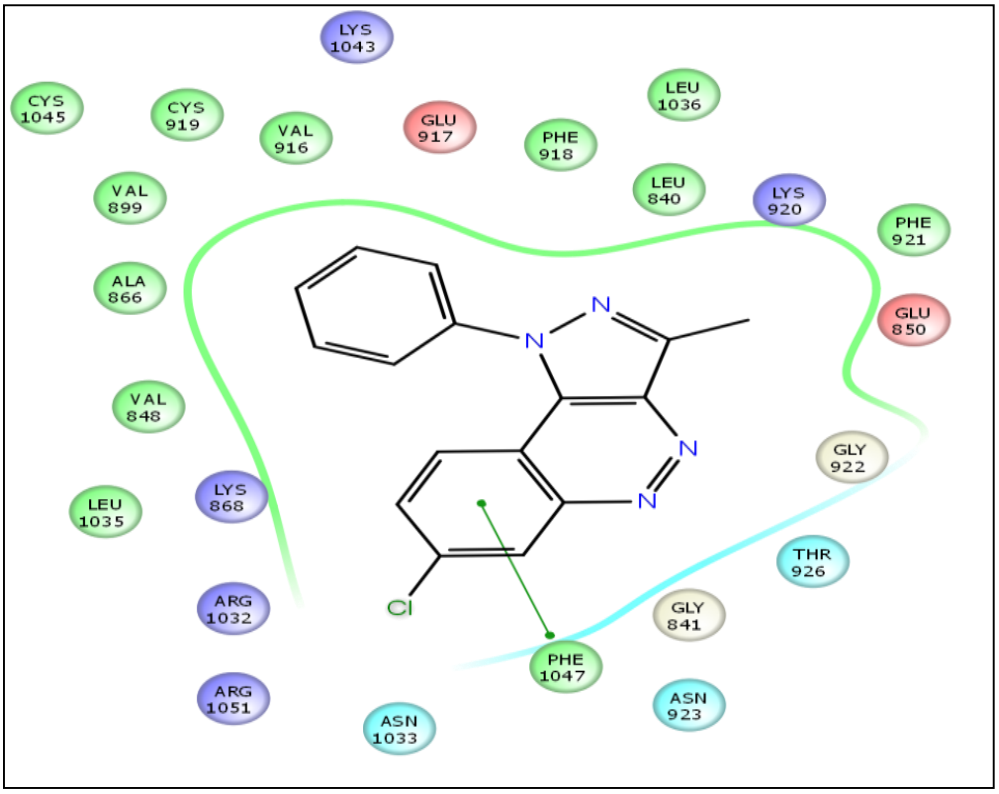


Figure 28: 3 D Interaction Of Compound PZ-2

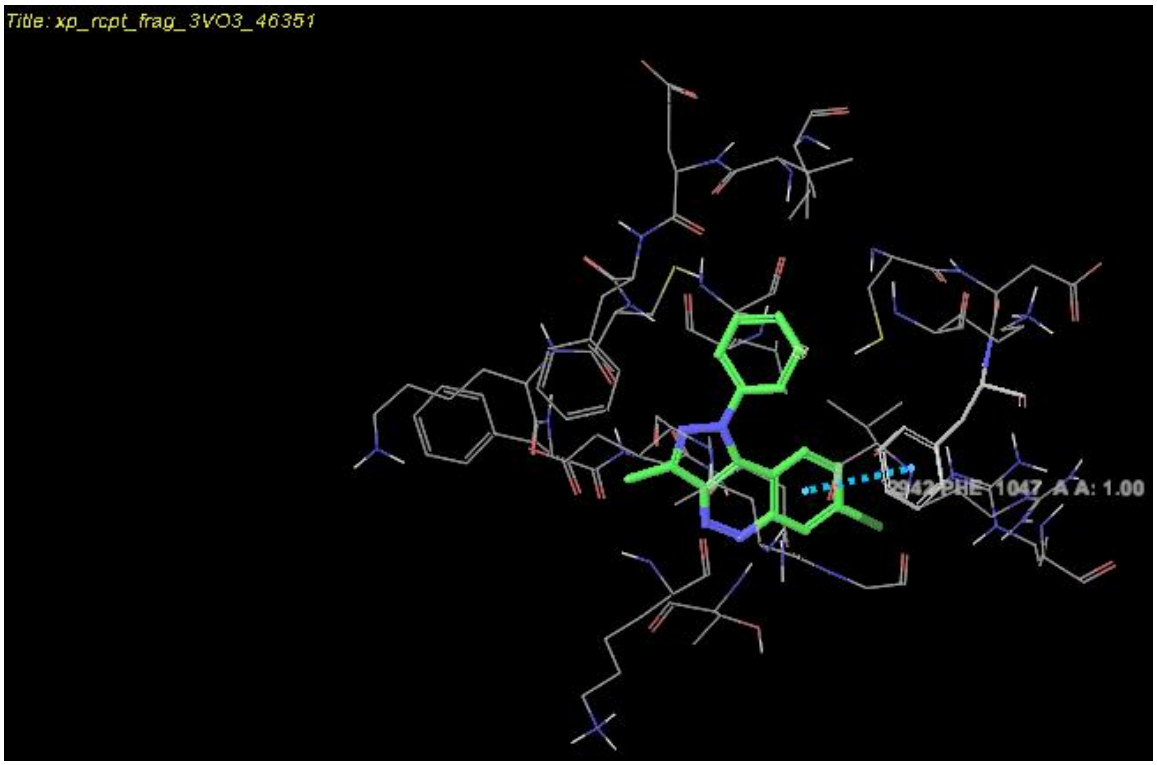


Figure 29: 2 D Interaction Of Compound PZ-3

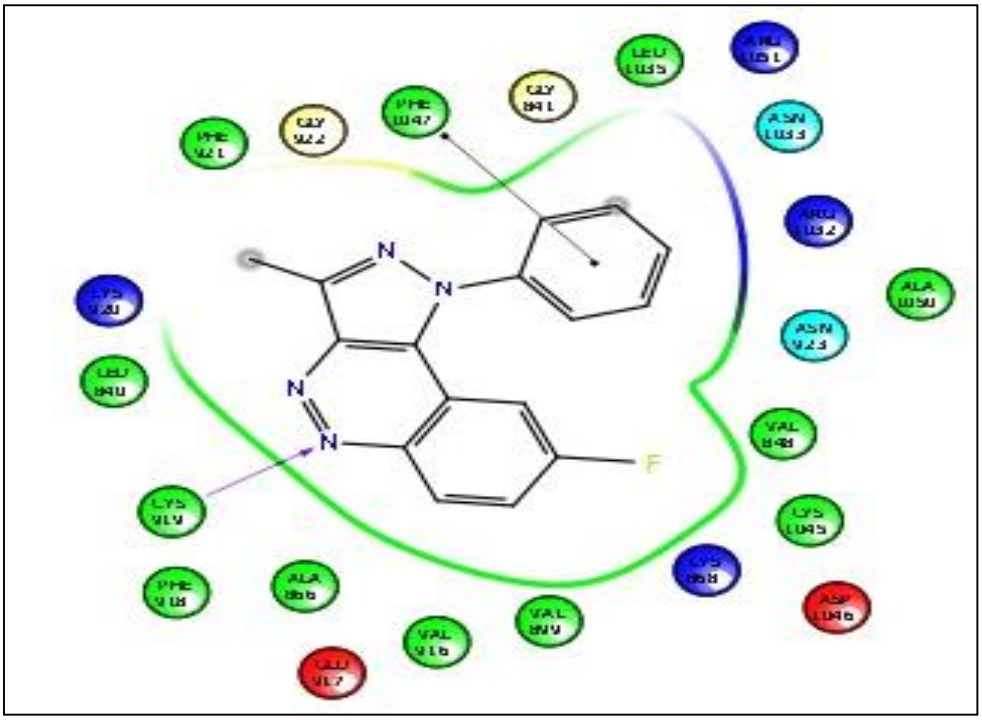


Figure 30: 2 D Interaction Of Compound PZ-4

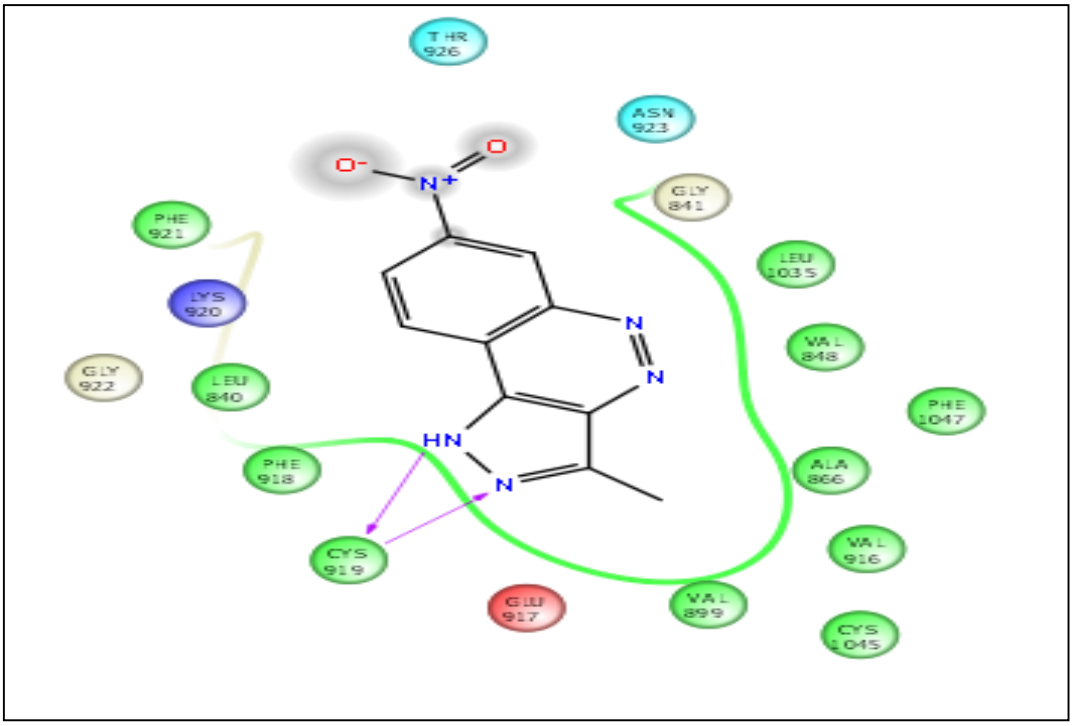


Figure 31: 2 D Interaction Of Compound PZ-5

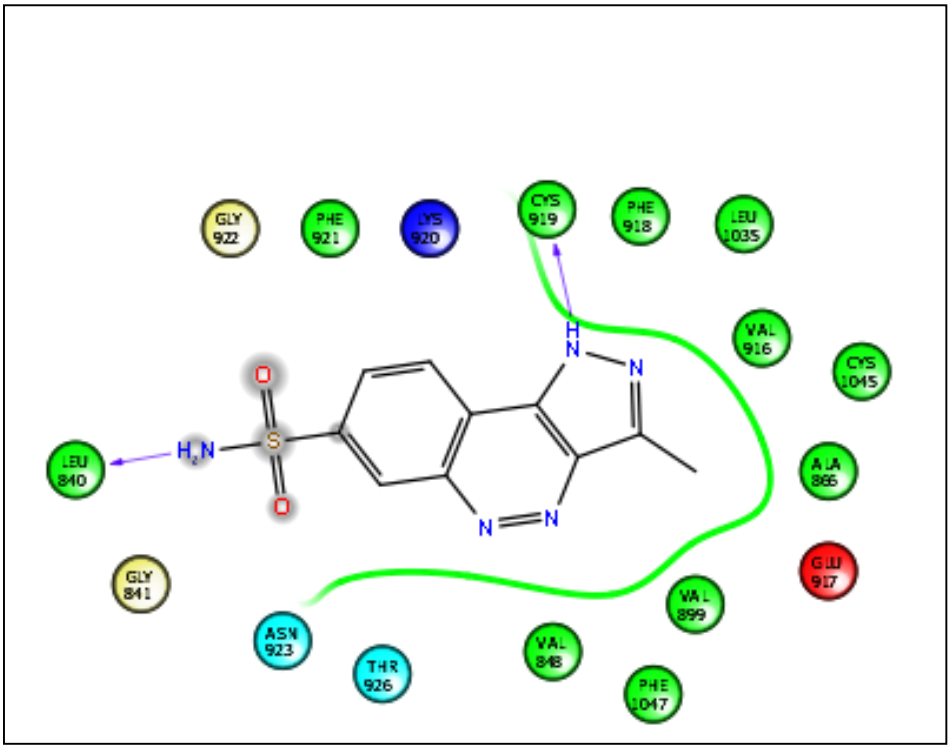


Figure 32: 3 D Interaction Of Compound PZ-5

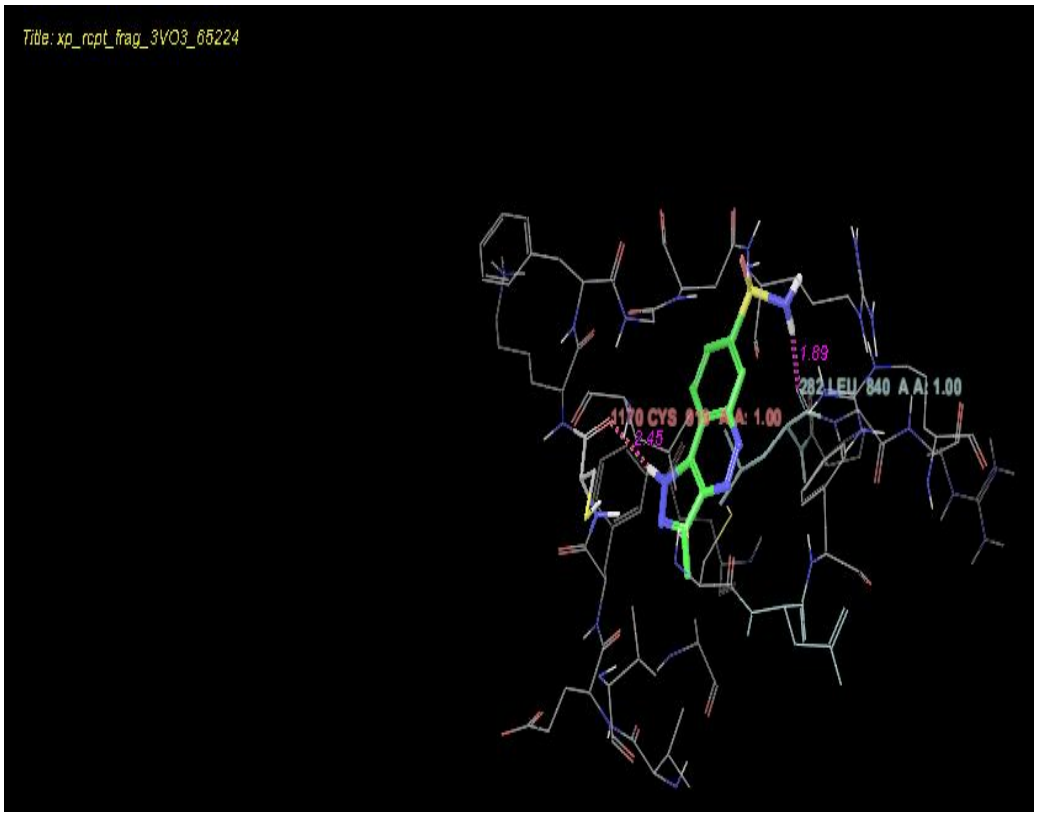


Figure 33: 2 D Interaction Of Compound PZ-6

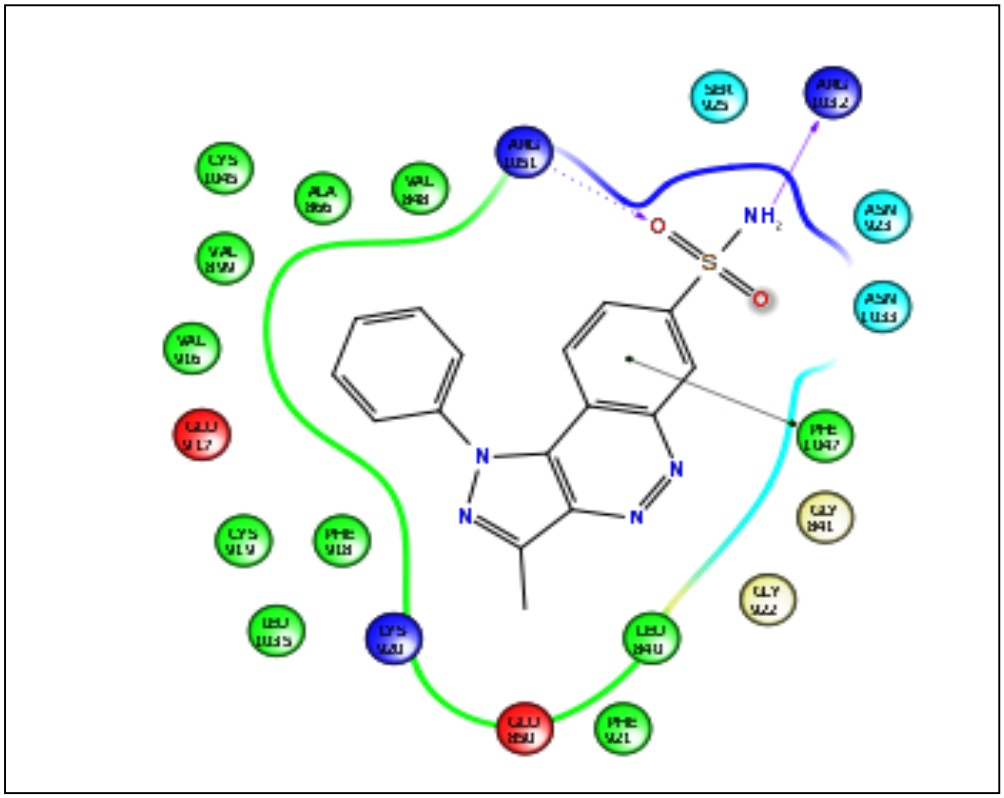
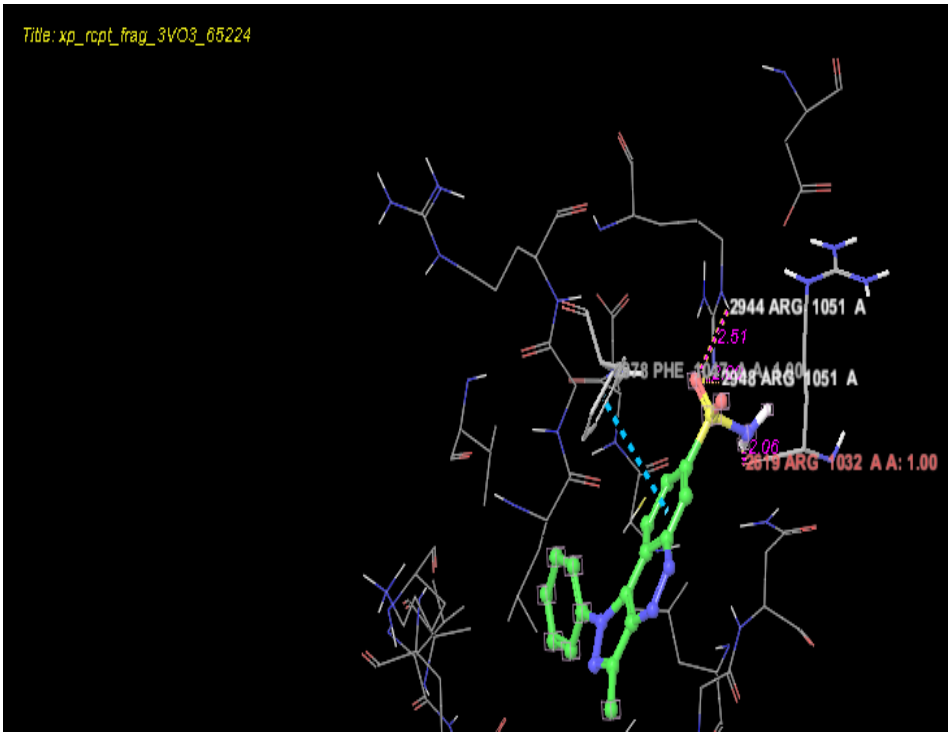


Figure 34: 3 D Interaction Of Compound PZ-6



SYNTHESIS OF PYRIDAZINE DERIVATIVES

The novel Benzpyridazine derivatives were designed on the basis of literature survey . Several compounds were hypothetically designed and performed molecular modelling studies by using Glide software. Based on the docking scores and feasibility of synthesis, 6 compounds were selected and synthesized by various reaction intermediates such as

Step 1: Synthesis of Ethyl 3 –oxo-2-(2-substituted phenylhydrazinylidene) butanoate.

Step 2: Synthesis of 3-Acetyl benzpyridazine-4(1*H*)-one derivatives.

Step 3: Synthesis of Pyrazolo benzpyridazine derivatives.

Physicochemical Studies of Synthesised Compounds

Physical parameters like percentage yield, molecular weight, molecular formula, melting point, logP and $R_f$value of the synthesized compounds were determined and is given in table 19. The final compounds were in moderate to higher yields. The percentage yield was found to be in the range of 70 – 95 %.

Table 19: Physicochemical parameters of synthesised compounds

Compound Code	Molecular Formula	Molecular Weight	% Yield	Colour	Solubility	M.P ^oC	R^f value*
PZ-1	$C_{10}H_7ClN_4$	218.65	74.5	Orange	DMSO	232	0.76
PZ-2	$C_{16}H_{11}ClN_4$	294.75	95.6	Orange	DMSO	180	0.42
PZ-3	$C_{16}H_{11}FN_4$	278.29	92.3	Orange	DMSO	200	0.96
PZ-4	$C_{10}H_7N_5O_2$	305.30	78.68	Brown	DMSO	100	0.61
PZ-5	$C_{10}H_9N_5SO_2$	263.28	88.3	Yellow	DMSO	210	0.21
PZ-6	$C_{16}H_{13}N_5SO_2$	339.38	94.32	Yellow	DMSO	200	0.73

***** Mobile phase – Chloroform : Methanol -0.2:9.8

SPECTRAL STUDIES OF SYNTHESISED COMPOUNDS

The structure of the synthesised compounds were compounds were confirmed by IR, NMR , and mass spectral analysis and is shown in table 20.The IR ,NMR and MASS spectra of synthesised compounds were shown in figure 34-50.

Table 20: Spectral analysis of synthesised compounds

Compound code	λmax (nm)	IR (cm^{-1})	^{1}H NMR (δ ppm)	^{13}C NMR (δ ppm)	MASS (m/z)
PZ-1	432	2911(CH stretching) 3162.69(NH stretching) 1588.59(N=N stretching) 1667(C=N) 1045.71(C-N) 900-675 (Aromatic group) 3162.69(Aromatic C-H Stretching) 1096 (C-Cl)	7.01-7.6 (Ar H) 2.15(H, CH3) 11.58(H, NH)	11.56(C of CH_3) 143.12-146.97(C =N pyrazole ring) 159.90 (C-C Cinnoline) 129.16(C-N Cinnoline) 114.41-143.12(C of Aromatic ring)	218.64
PZ-2	207.5	2924.04(Aromatic CH stretching) 3484.26(NH stretching) 1618.95(N=N stretching) 1468.05(C=N stretchimg) 1249.16(C-N stretching) 900-675 (Aromatic group) 3162.69(Aromatic C-H Stretching) 600-800 (C-Cl stretching)	7-7.48 (Aromatic H) 2.132(H in CH_3 group)	------	-------
PZ-3	272	2926.93(Aromatic CH stretching) 1614.13(N=N stretching) 1561.09(C=N str) 900-675 (Aromatic group) 1400-1000 (C-F stretching)	7-7.5 (Aromatic H) 2.23(H in CH_3 group)	-------	-------
Compound code	λmax (nm)	IR (cm^{-1})	^{1}H NMR (δ ppm)	^{13}C NMR (δ ppm)	MASS (m/z)

PZ-4	207.5	2921.63(Aromatic CH stretching) 3411.46(NH stretching) 1629.55(N=N stretching) 1405.37(C=N stretching) 613.735(Aromatic group)	10.69-11.29(H in NH group) 7.247-7.97(Aromatic H group) 2.022(H in CH_3 group	-------	-------
PZ-5	401.5	2921.63(CH stretching) 3411.94(NH stretching) 1617.5(N=N stretching) 1410.67(C=N stretching) 1160.45(C-N) 900-675(Aromatic group) 3302.02(C-SO_2NH_2 group) 1334.5(SO_2 stretching)	11.618(H in NH group) 7.46-7.99 (Aromatic H group) 2.17(H in CH_3 group	11.612(C of CH_3) 112-145.5(C of Aromatic ring) 129.306(C-N Cinnoline) 146.902 (C =N of pyrazole ring) 159.993(C-C Cinnoline**)**	263.75
PZ-6	272.5	2926.93(Aromatic CH stretching) 3450-3390(NH stretching) 1617.98(N=N stretching,C=N stretching) 1160.45(C-N) 900-675(Aromatic group) 3228.74(Aromatic C-SO_2 NH_2 group) 1332.57(SO_2 stretching)	7.23-7.93 (Aromatic H group) 2.33(H in CH_3 group	11.648(C of CH_3) 114.47-145.513(C of Aromatic ring) 129.0346(C-N Cinnoline) 145.513 (C =N of pyrazole ring) 156.327(C-C Cinnoline**)**	-------

Figure 35: IR Spectrum Of Compound PZ-1

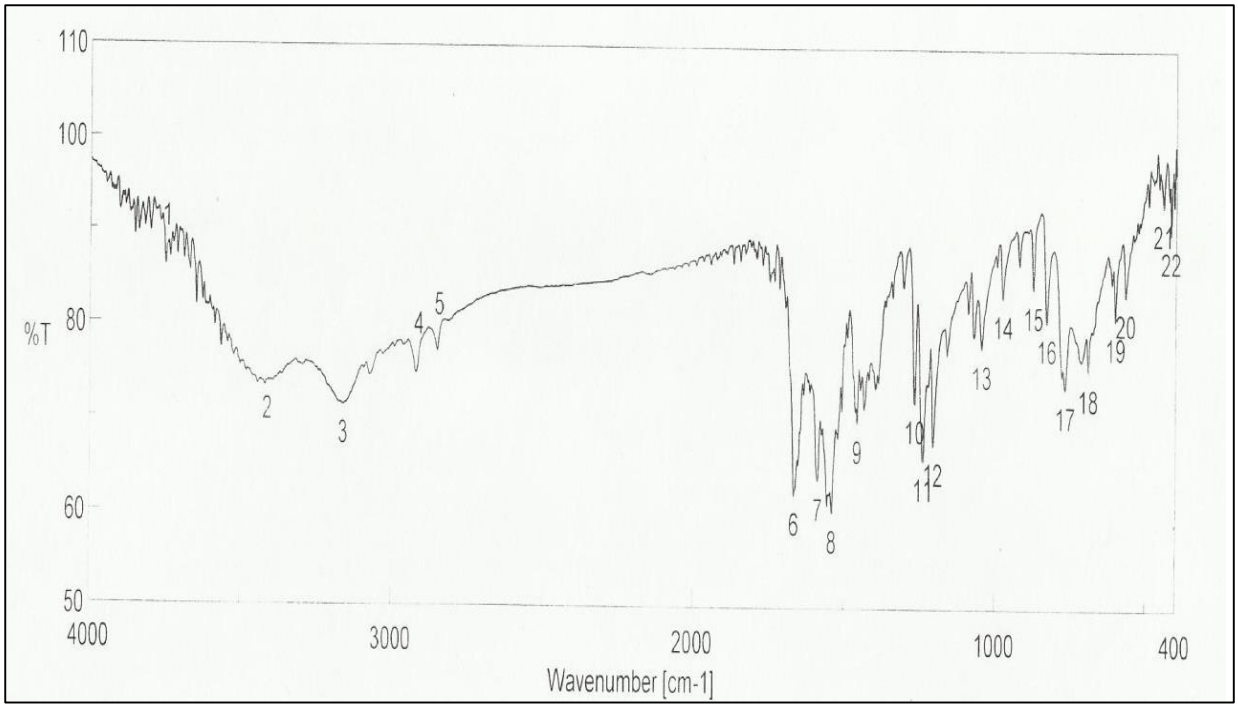


Figure 36: IR Spectrum Of Compound PZ-2

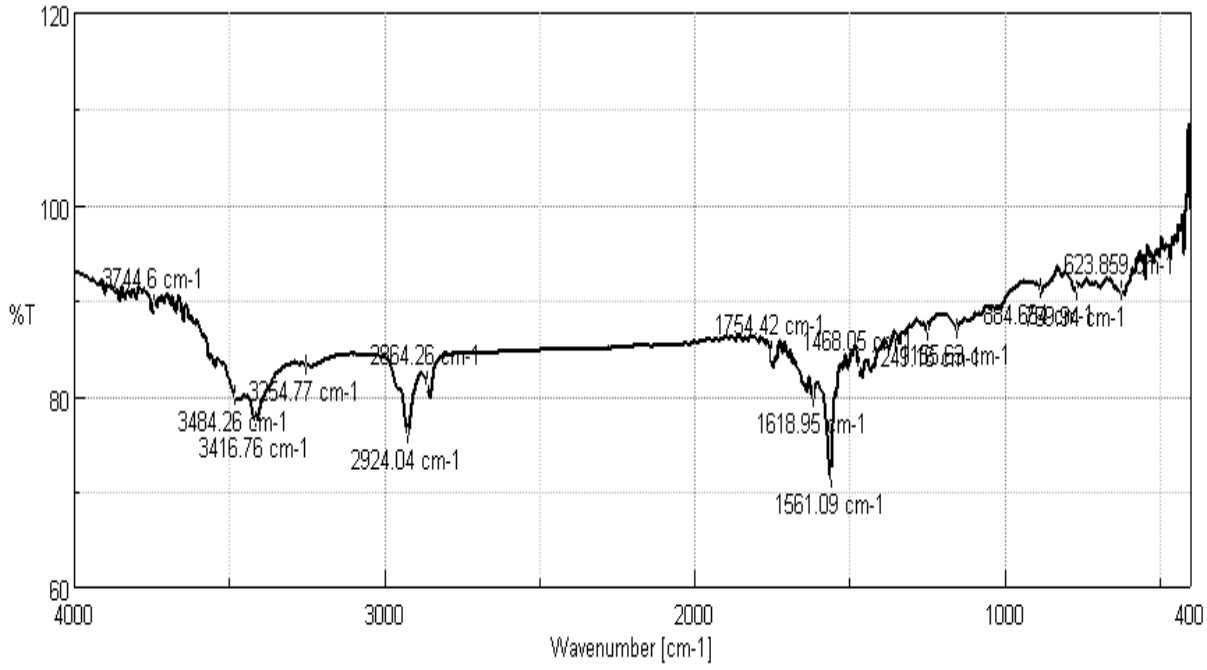


Figure 37: IR Spectrum Of Compound PZ-3

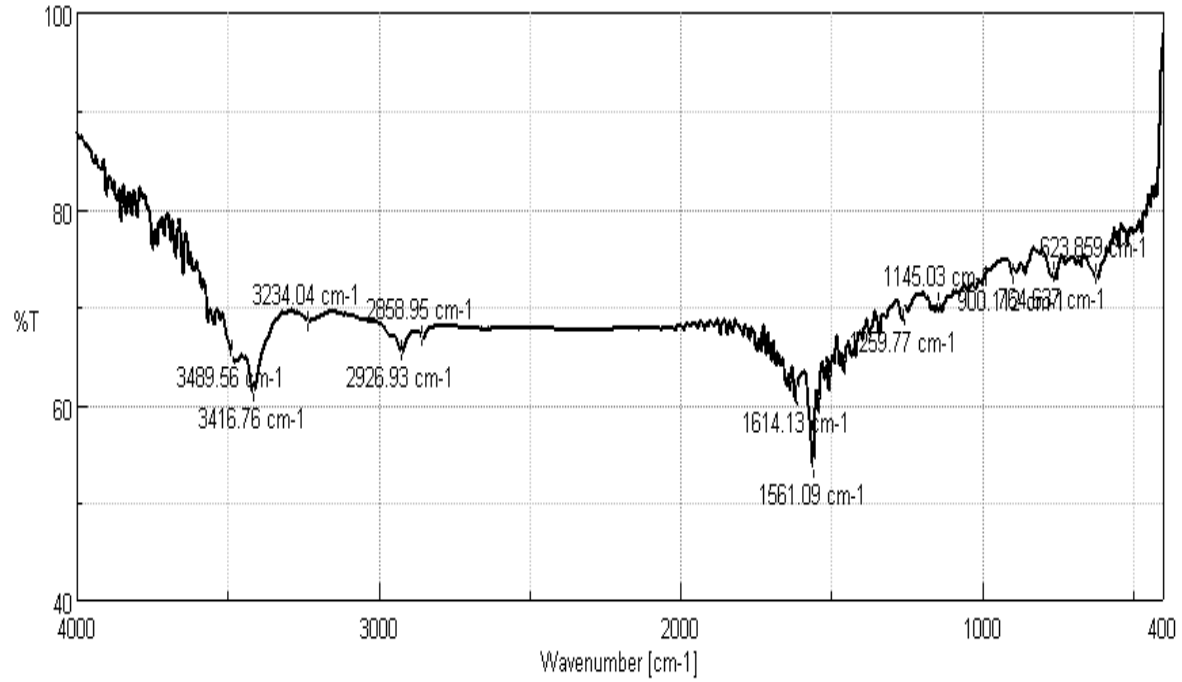


Figure 38: IR Spectrum Of Compound PZ-4

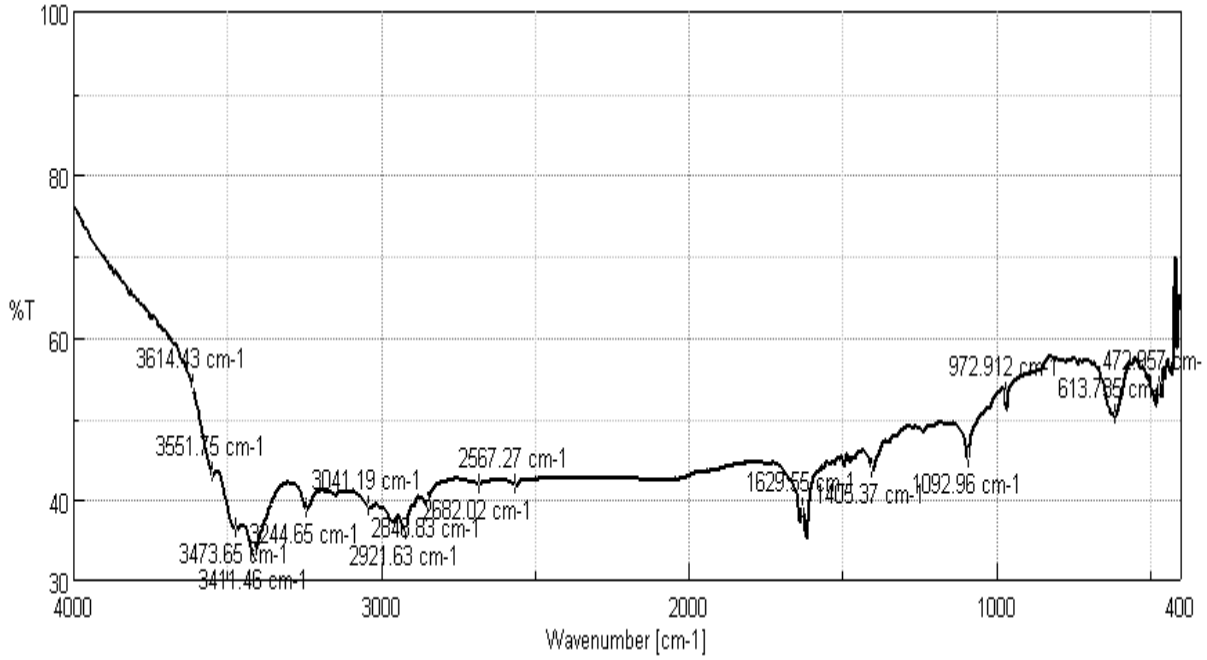


Figure 39: IR Spectrum Of Compound PZ-5

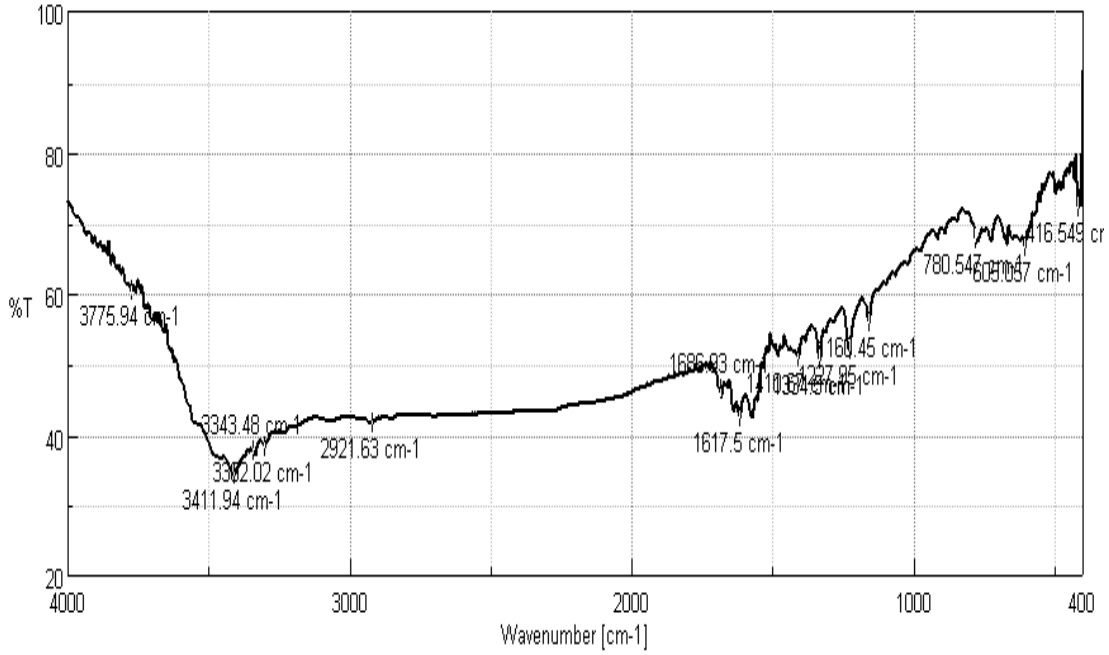


Figure 40: IR Spectrum Of Compound PZ-6

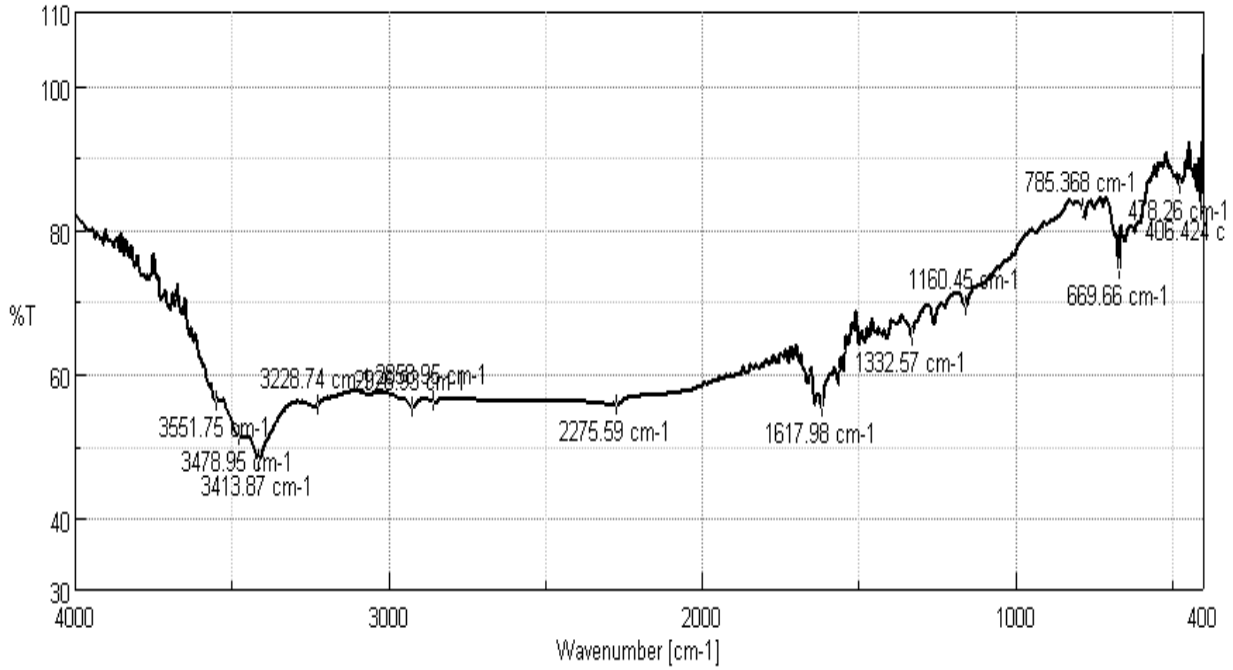


Figure 41: ^{1}H NMR Spectrum Of Compound PZ-1

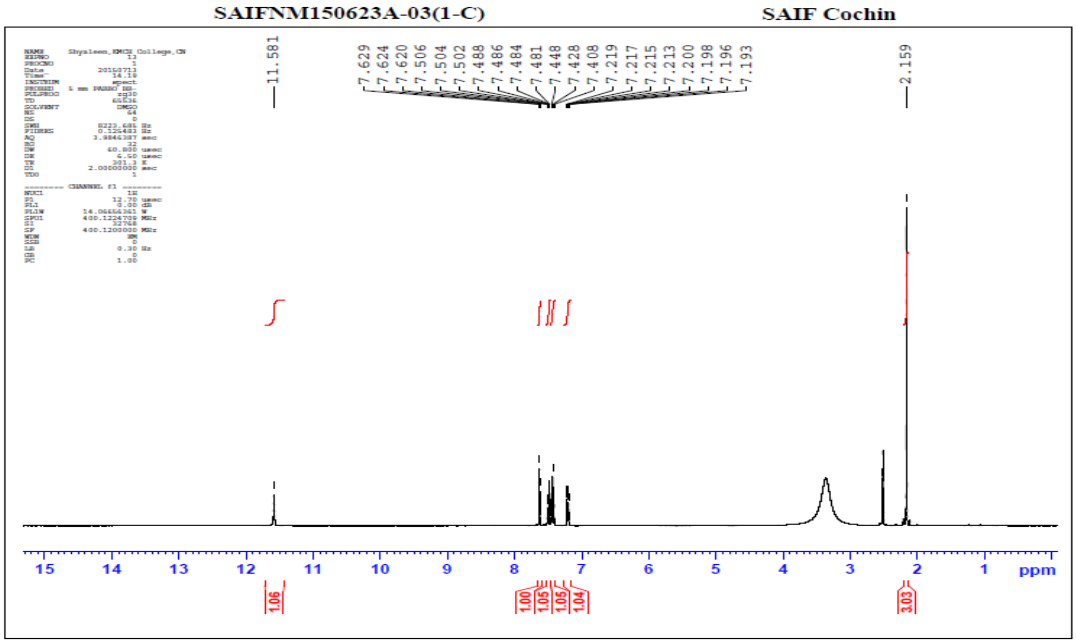


Figure 42: ^{13}C NMR Spectrum Of Compound PZ-1

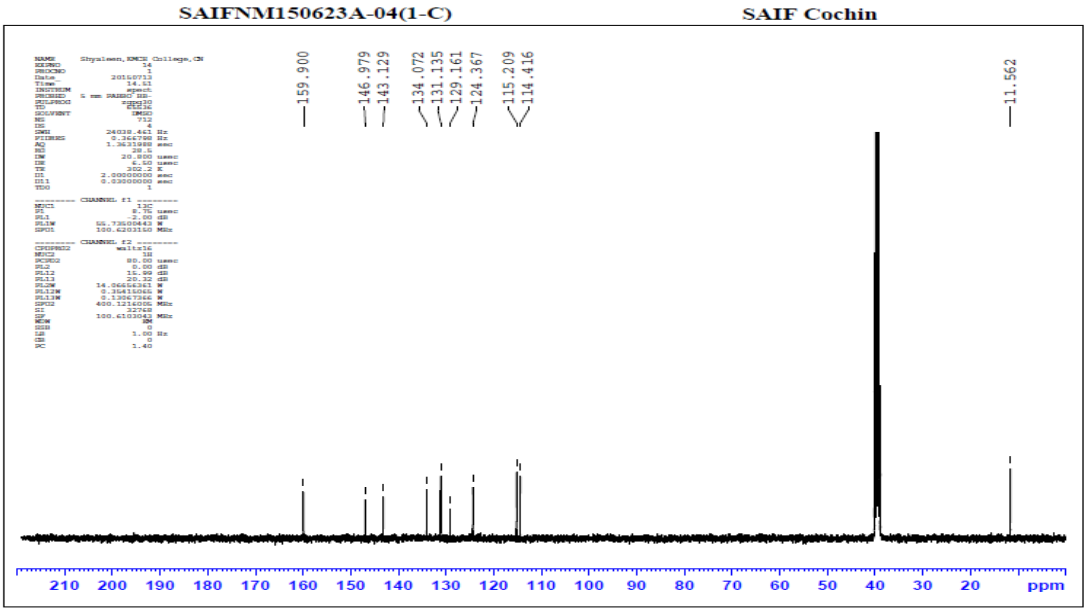


Figure 43: Mass Spectrum Of Compound PZ-1

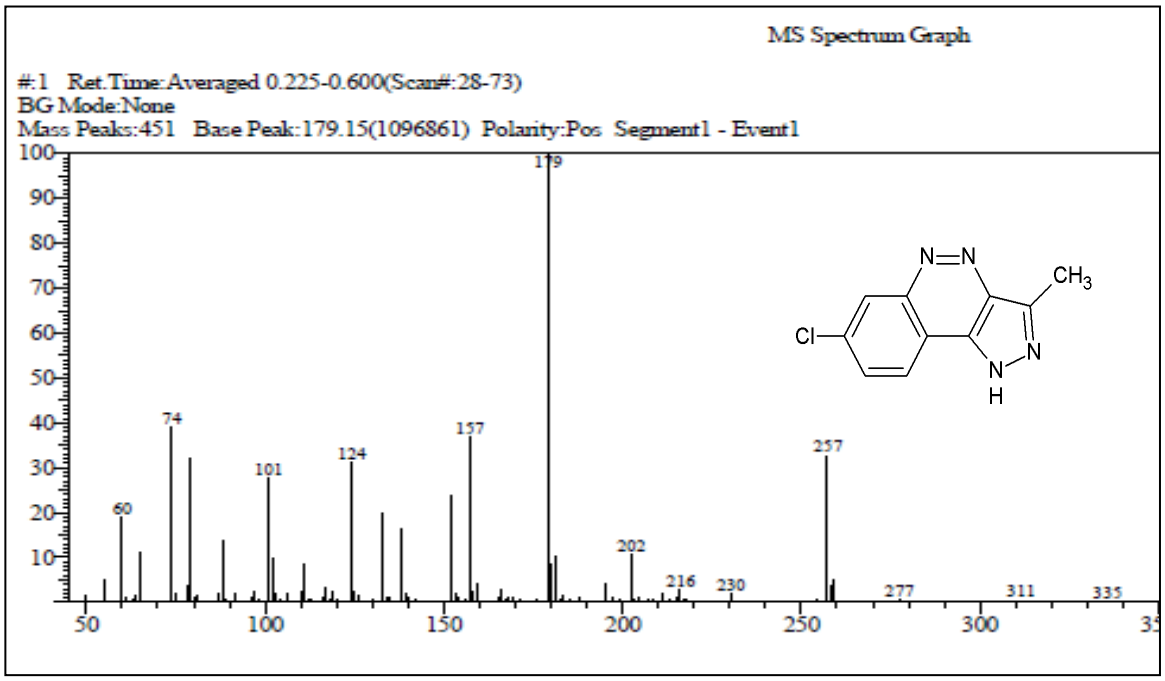


Figure 44: ^{1}H NMR Spectrum Of Compound PZ-2

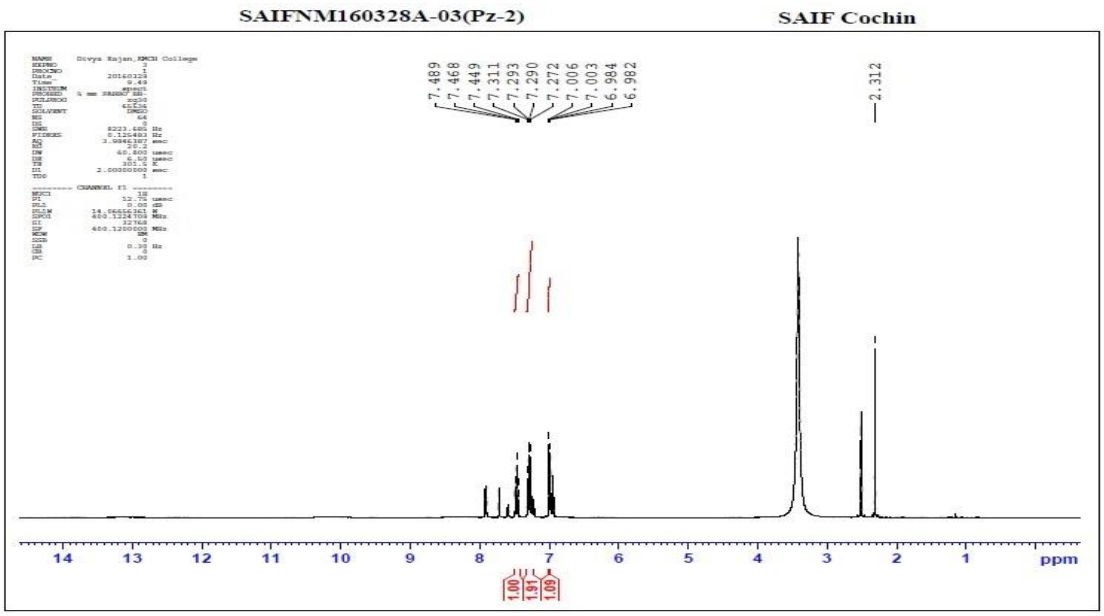


Figure 45: ^{1}H NMR Spectrum Of Compound PZ-3

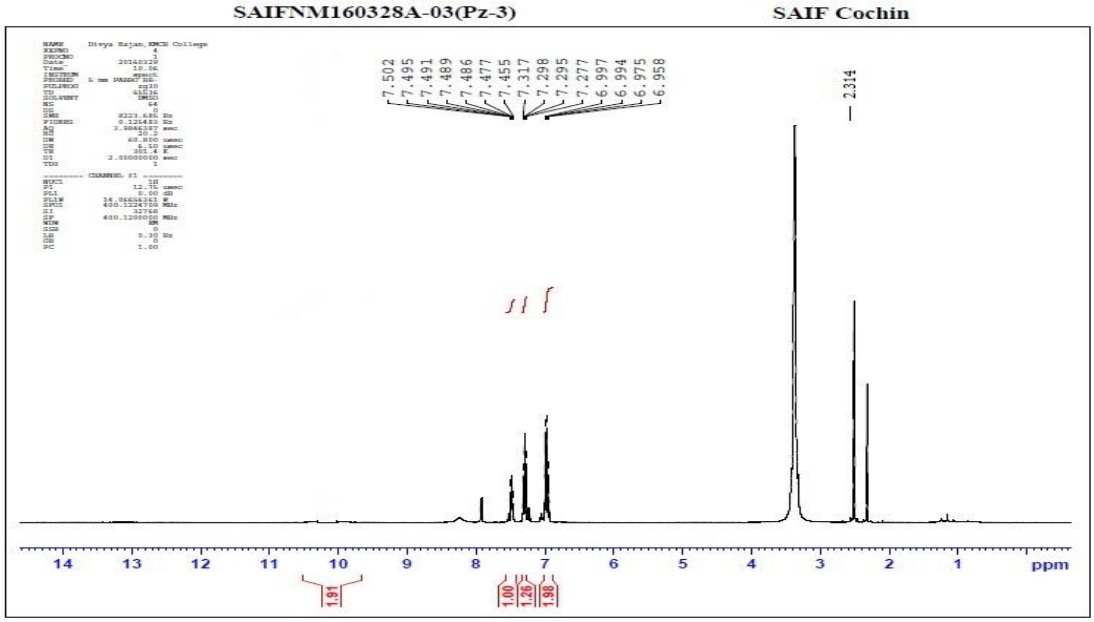


Figure 46: ^{1}H NMR Spectrum Of Compound PZ-4

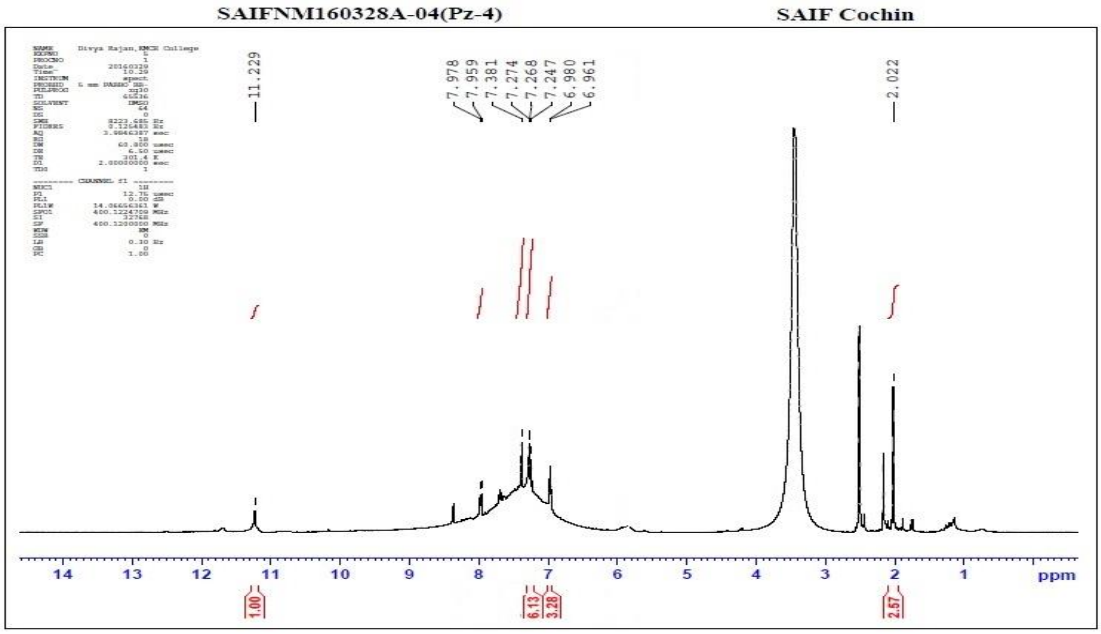


Figure 47: ^{1}H NMR Spectrum Of Compound PZ-5

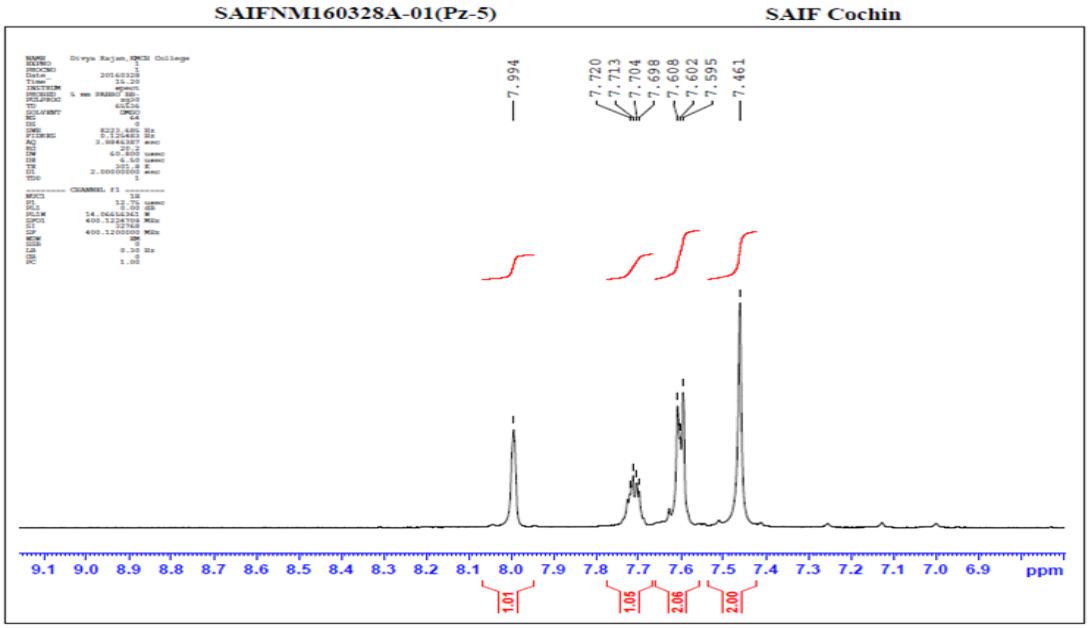


Figure 48: ^{13}C NMR Spectrum Of Compound PZ-5

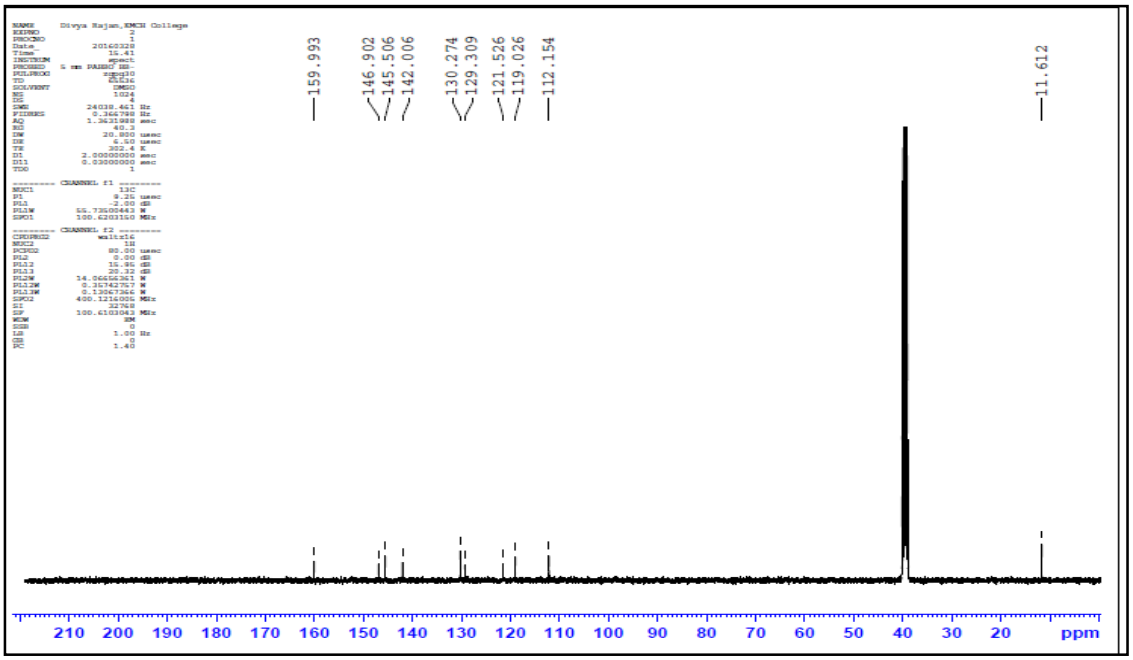


Figure 49: Mass Spectrum of Compound PZ-5

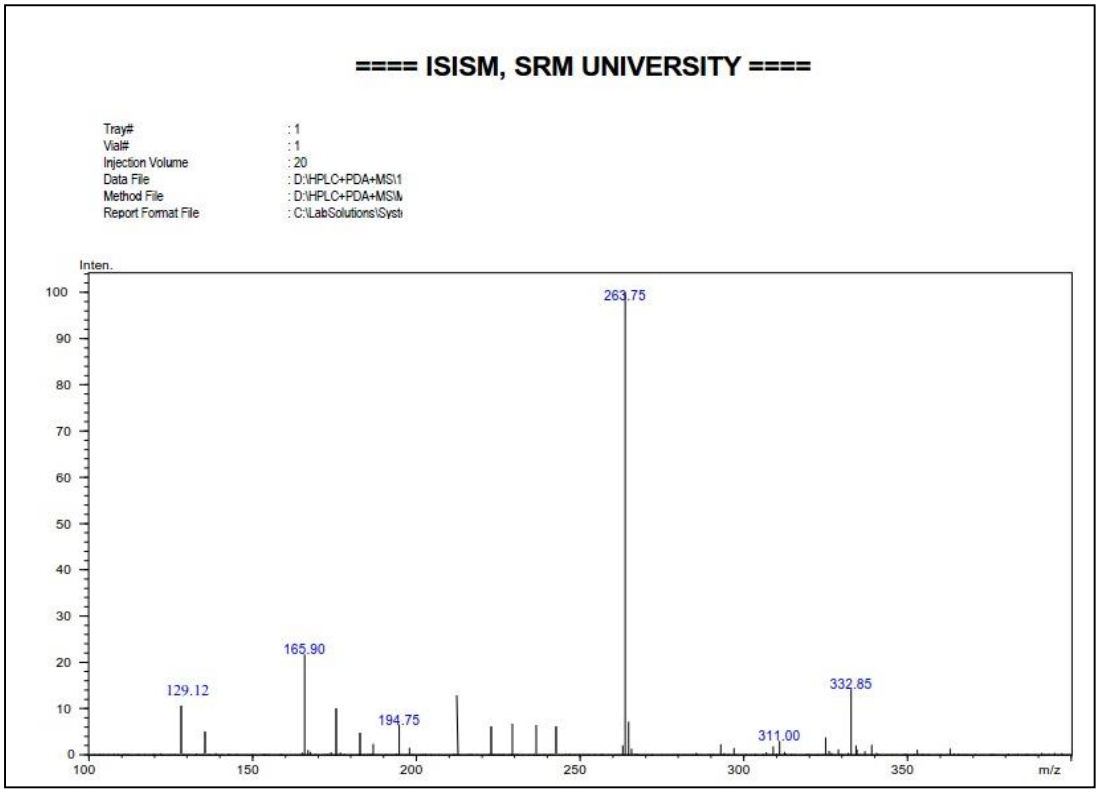


Figure 50: ^{1}H NMR Spectrum Of Compound PZ-6

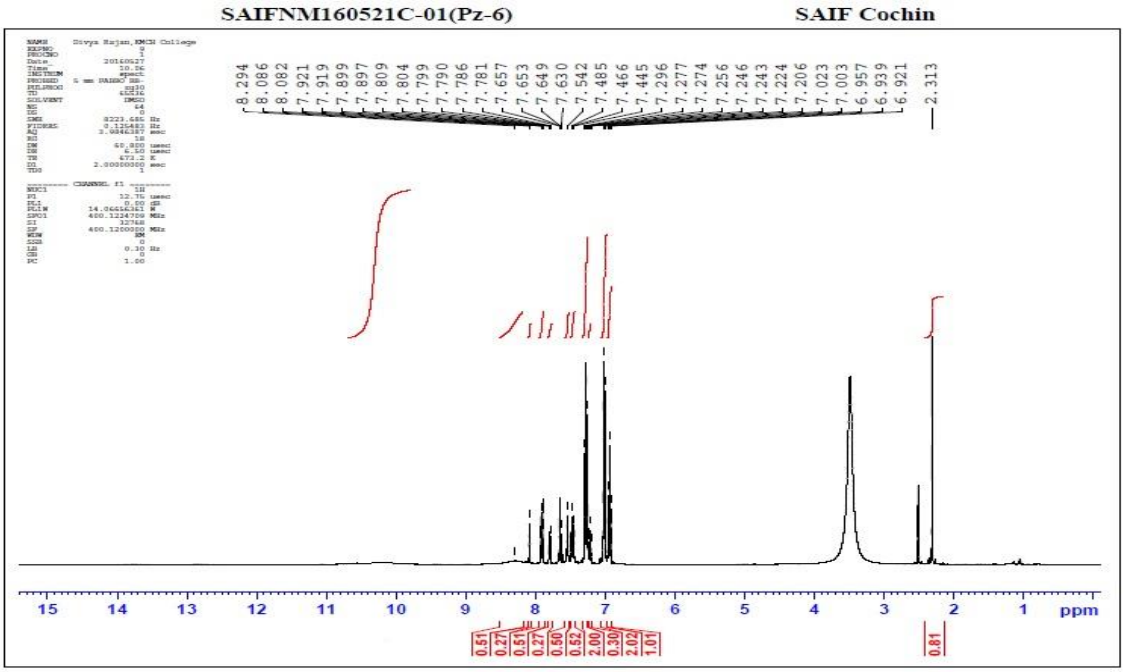
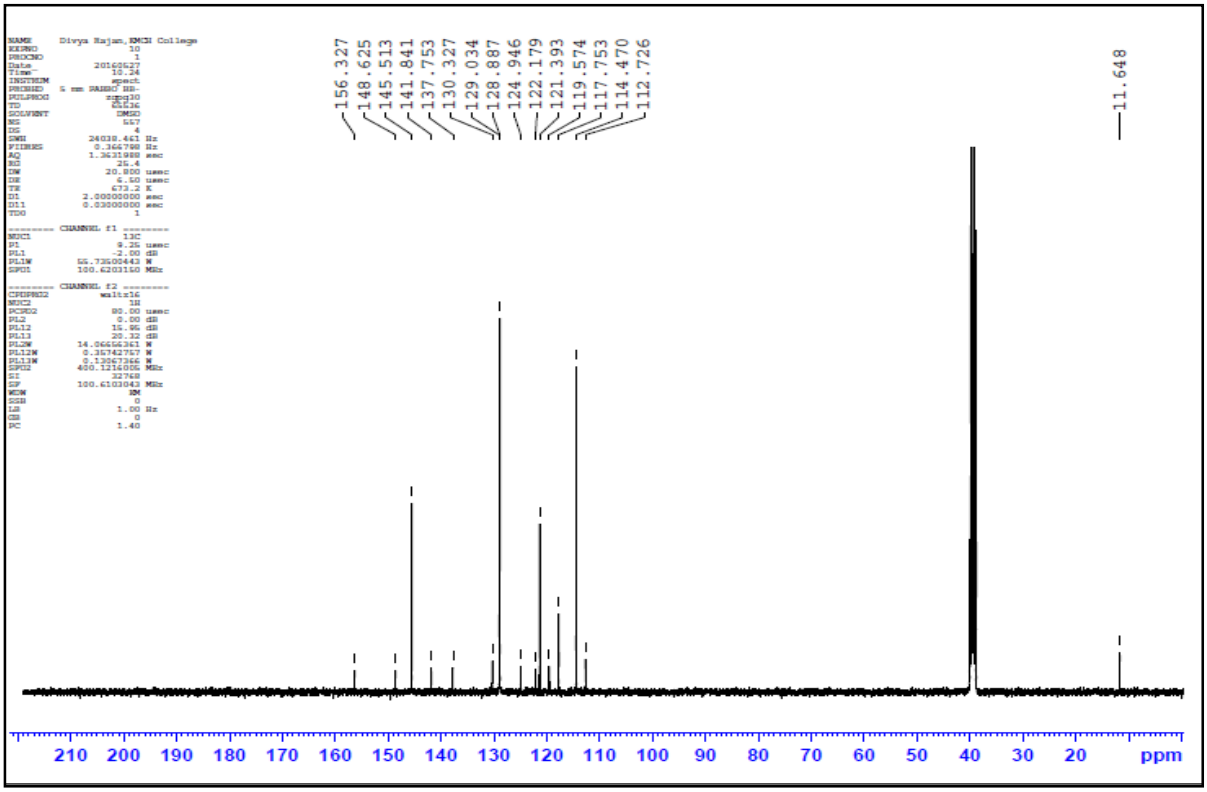


Figure 51: ^{13}C NMR Spectrum Of Compound PZ-6



IN VITRO STUDIES

In Vitro Antioxidant Activity

DPPH Radical Scavenging Activity

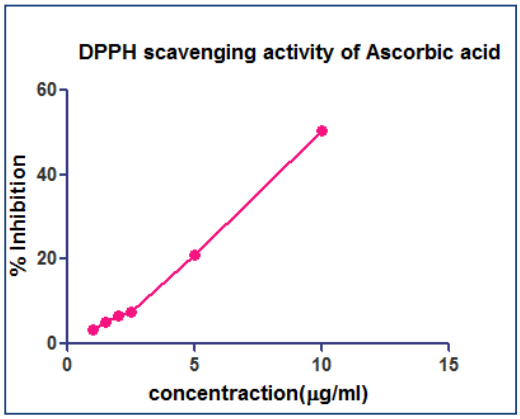
DPPH and ABTS antioxidant activity were performed in synthesised compounds and they showed good antioxidant activity**.** The percentage inhibition and IC_{50} values are given in table 21 and DPPH radical scavenging activity of ascorbic acid and the synthesised compounds are shown in figure 51-52.

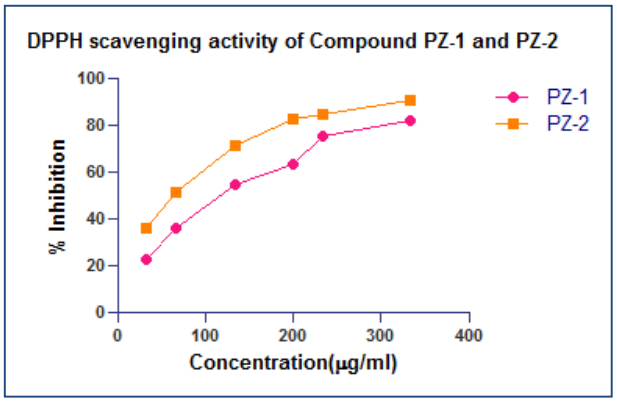
Table 21 : Percentage inhibition and IC_{50} values of DPPH radical by Ascorbic acid and the synthesised compounds.

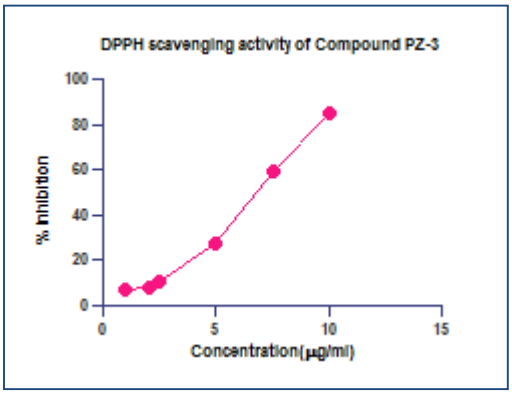
Sample	Concentration (µg/ml)	% Inhibition	IC_{50} (µg/ml)
Standard(Ascorbic acid)	1.0	3.37	10.10
	1.5	5.08	
	2.0	6.66	
	2.5	7.60	
	5.0	20.98	
	10.0	50.36	
PZ-1	33.33	22.81	106.4
	66.66	35.87	
	133.33	54.90	
	200.00	63.11	
	233.33	75.18	
	333.33	82.12	
PZ-2	33.33	35.83	58.55
	66.66	51.47	
	133.33	71.31	
	200.00	82.88	
	233.33	84.92	
	333.33	90.53	
PZ-3	1.0	6.71	6.469
	2.0	7.81	
	2.5	10.47	
	5.0	27.45	
	7.5	59.32	
	10.0	85.22	
PZ-4	33.33	45.64	43.20
	66.66	55.57	
	133.33	84.16	
	200.00	86.82	
	233.33	87.73	
	333.33	91.00	

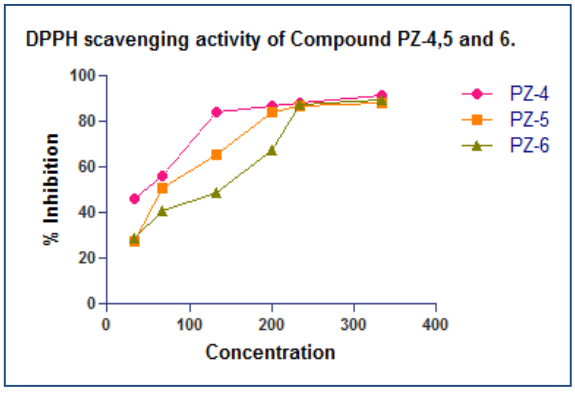
Sample	Concentration (µg/ml)	% Inhibition	IC_{50} (µg/ml)
PZ-5	33.33	27.40	68.72
	66.66	50.63	
	133.33	65.25	
	200.00	84.16	
	233.33	86.82	
	333.33	87.73	
PZ-6	33.33	28.41	91.90
	66.66	40.73	
	133.33	48.35	
	200.00	67.14	
	233.33	86.95	
	333.33	89.05	

Figure 52: DPPH radical scavenging activity of Ascorbic acid and the synthesised compounds.







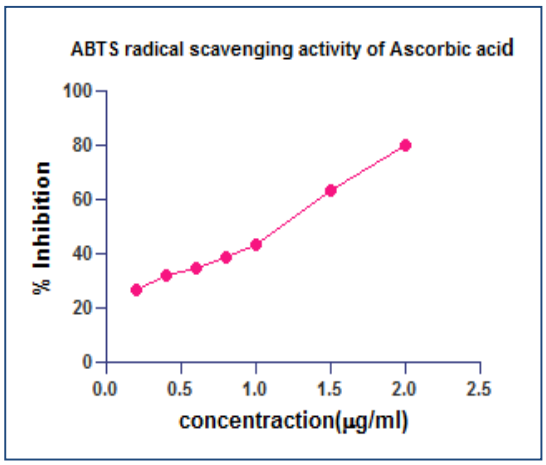


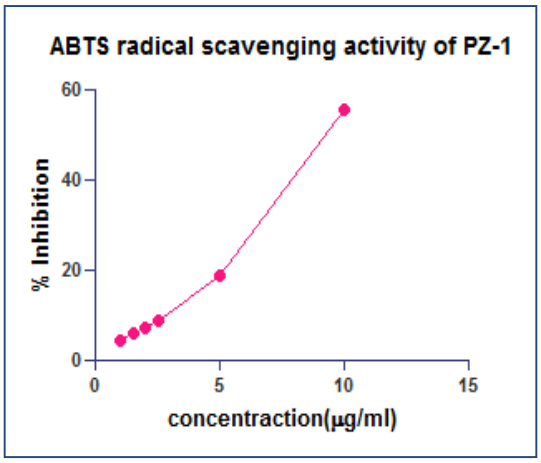
ABTS RADICAL SCAVENGING ACTIVITY

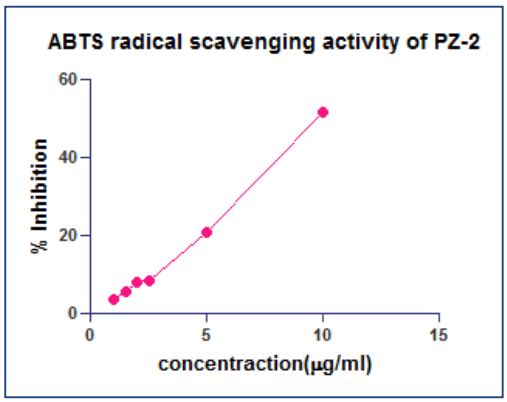
Table 22: Percentage inhibition of ABTS radical by Ascorbic acid and the synthesised compounds.

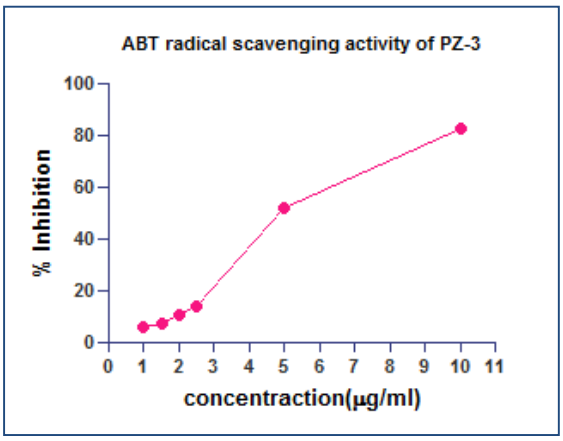
Sample	Concentration (µg/ml)	% Inhibition	IC_{50} (µg/ml)
Standard(Ascorbic acid)	0.2	26.79	0.9374
	0.4	32.17	
	0.6	34.94	
	0.8	38.64	
	1.0	43.49	
	1.5	63.04	
	2.0	80.22	
PZ-1	1.0	4.23	9.263
	1.5	6.04	
	2.0	7.23	
	2.5	8.66	
	5.0	18.56	
	10.0	55.66	
PZ-2	1.0	3.45	10.01
	1.5	5.36	
	2.0	7.68	
	2.5	8.32	
	5.0	10.53	
	10.0	51.65	
PZ-3	1.0	5.85	4.977
	1.5	6.86	
	2.0	10.56	
	2.5	13.65	
	5.0	60.35	
	10.0	72.88	
PZ-4	1.0	6.90	10.49
	1.5	8.46	
	2.0	11.57	
	2.5	14.66	
	5.0	19.67	
	10.0	51.84	
PZ-5	1.0	5.23	4.883
	1.5	7.34	
	2.0	9.56	
	2.5	15.85	
	5.0	58.65	
	10.0	75.65	
PZ-6	1.0	6.50	8.849
	1.5	8.46	
	2.0	11.46	
	2.5	16.45	
	5.0	35.46	
	10.0	52.46	

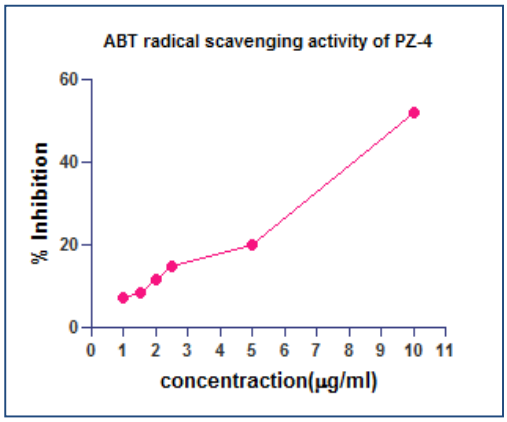
Figure 53: ABTS radical scavenging activity of Ascorbic acid and the synthesised compounds.

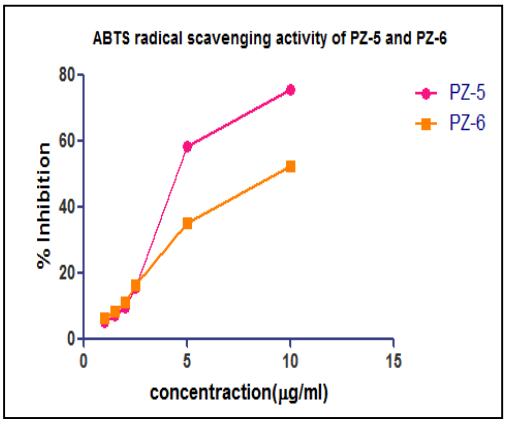












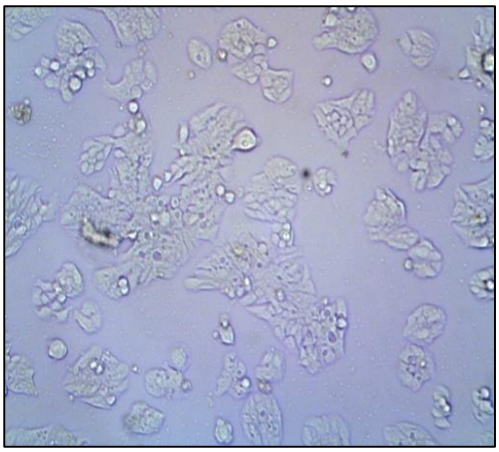
IN VITRO STUDIES OF SYNTHESISED COMPOUNDS

In order to confirm the activity of the synthesised compounds against colon cancer, the compounds were tested for their cytotoxicity against colon cancer cell lines(HT-29) and is given in table 23.Of the tested compounds, the compound PZ-5 was found to possess significant cytotoxicity against the HT-29 cell line with an IC_{50} value of 27.64µM and the HT 29 cell inhibition of synthesised compounds were shown from fig.53-60.

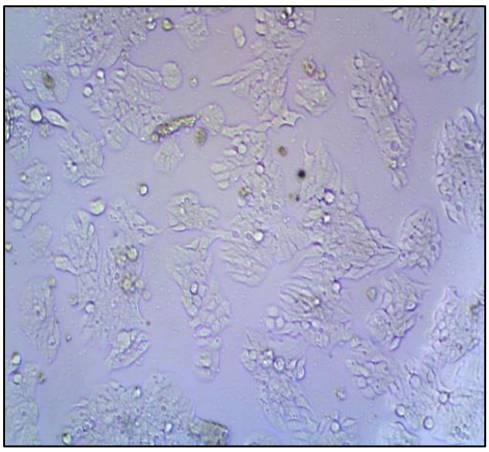
Table 23: HT-29 Cell Line Inhibition of Synthesised Compounds

Comp. code	Concentration	% cell inhibition	IC_{50}
Pz-1	0.25 2.5 25 50 100	0.084 0.126 2.763 6.028 25.409	>100
Pz-2	0.25 2.5 25 50 100	0.084 0.867 1.339 8.957 29.259	>100
Pz-3	0.25 2.5 25 50 100	0.627 4.185 31.268 37.881 52.867	85.89
Pz-4	0.25 2.5 25 50 100	0.085 0.596 1.233 5.544 16.367	>100
Pz-5	0.25 2.5 25 50 100	13.436 33.235 46.672 57.722 59.690	27.64
Pz-6	0.25 2.5 25 50 100	0.098 0.698 2.689 6.941 26.846	>100

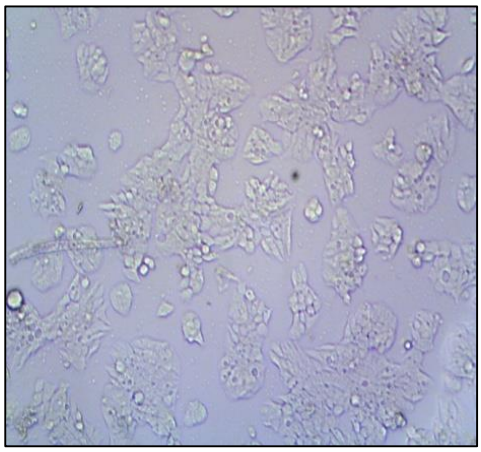
Figure 54: HT-29 Cell Line Inhibition By Compound PZ-1



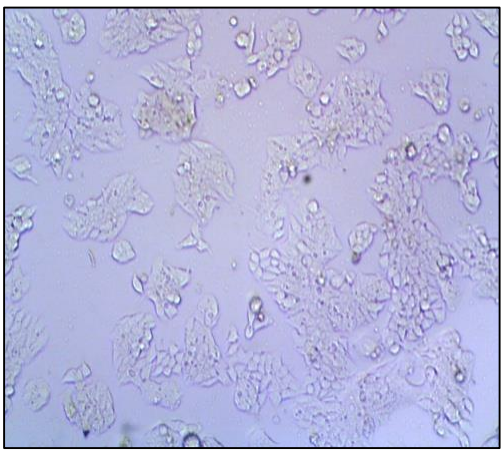
0.25 µM



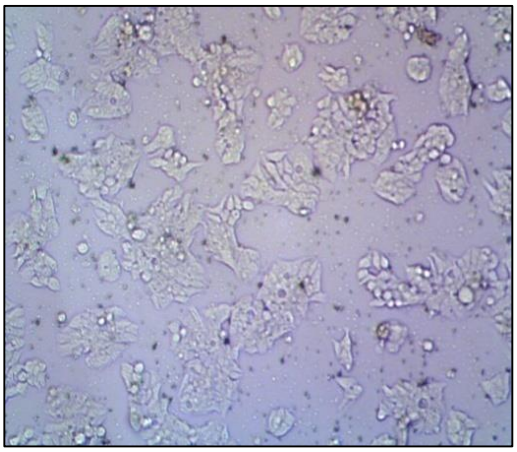
2.5 µM



25 µM

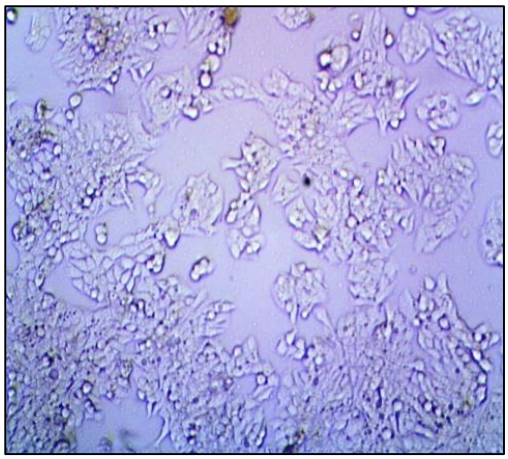


50 µM

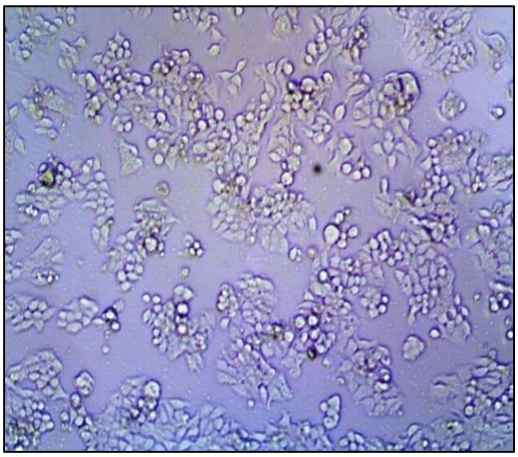


100µM

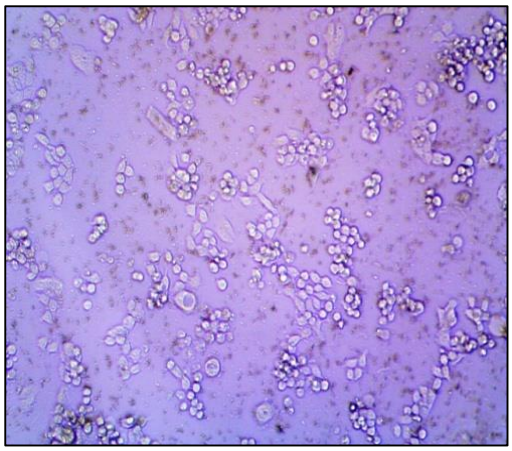
Figure 55: HT-29 Cell Line Inhibition By Compound PZ-2



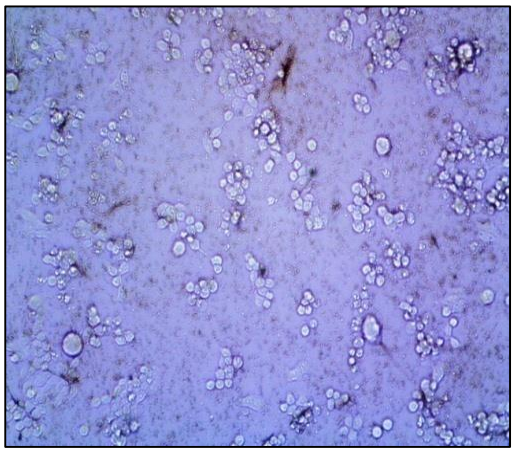
0.25 µM



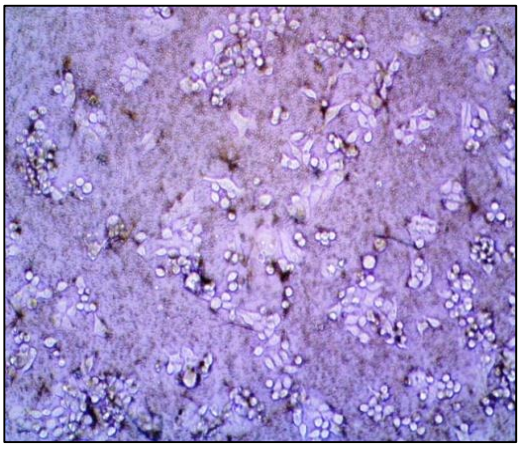
2.5 µM



25 µM

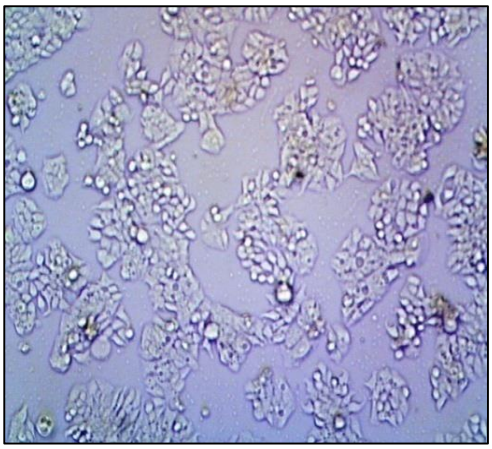


50 µM

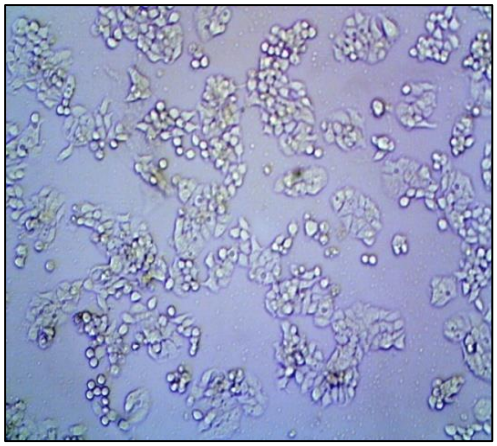


100 µM

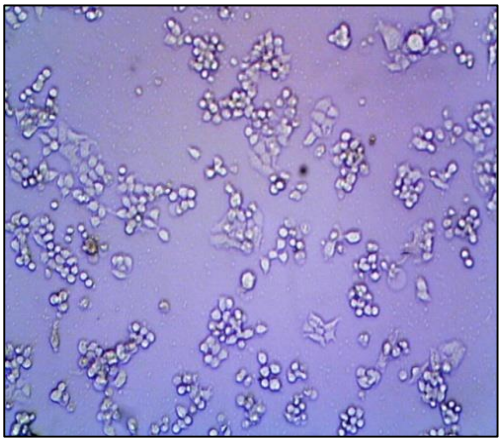
Figure 56: HT-29 Cell Line Inhibition By Compound PZ-3



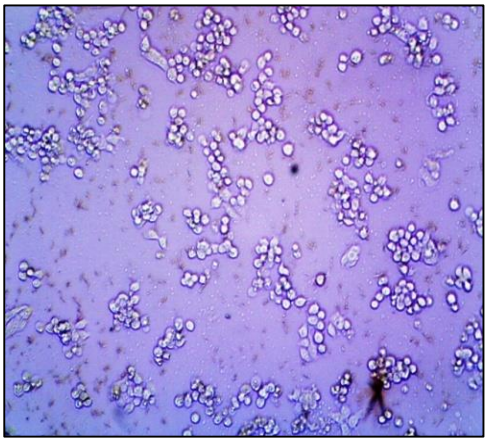
0.25 µM



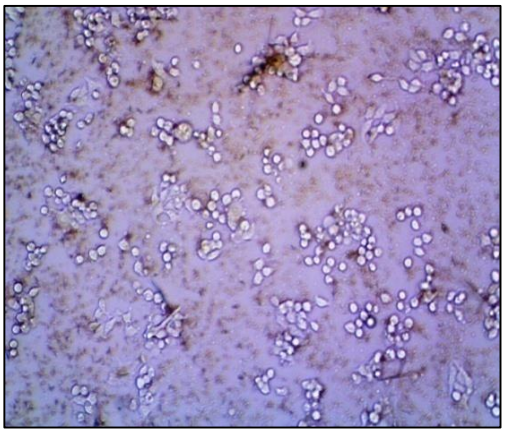
2.5 µM



25 µM

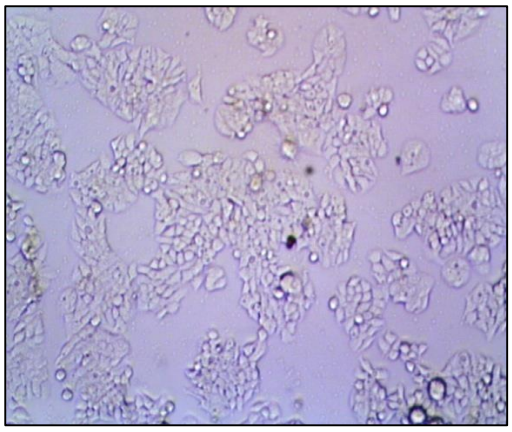


50 µM

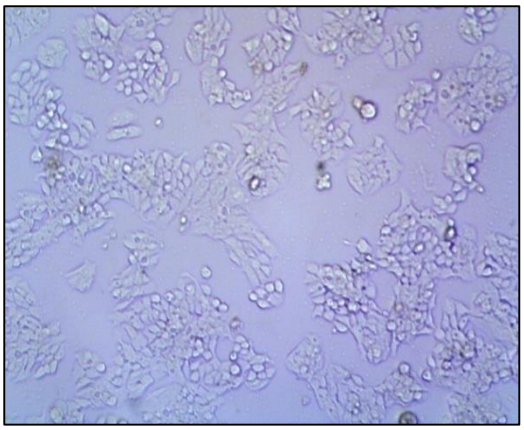


100 µM

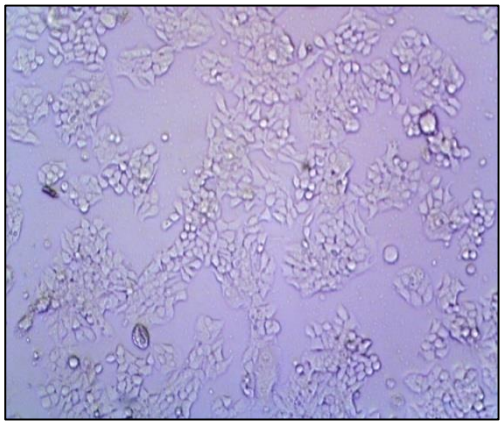
Figure 57: HT-29 Cell Line Inhibition By Compound PZ-4



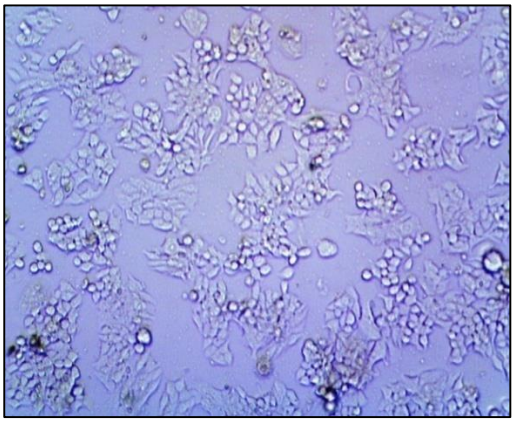
0.25 µM



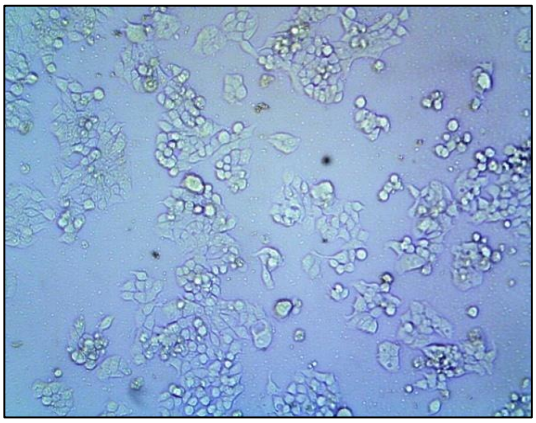
2.5 µM



25 µM

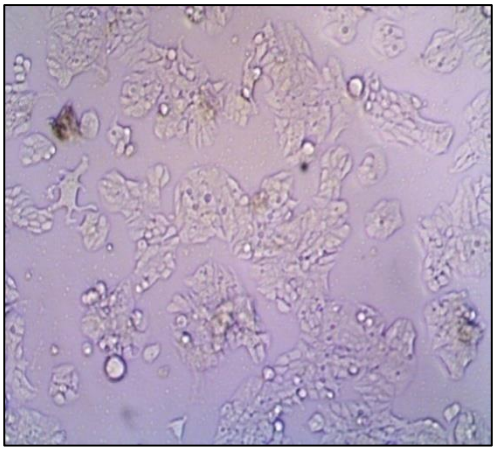


50 µM

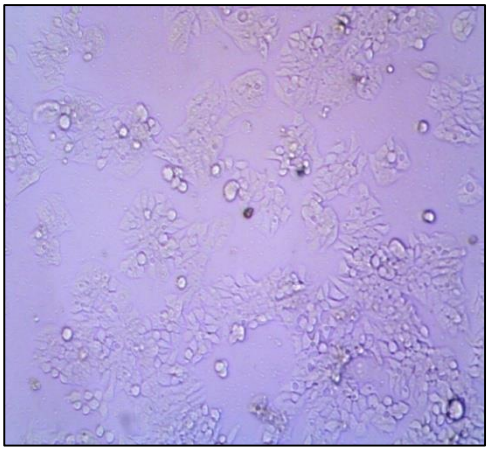


100 µM

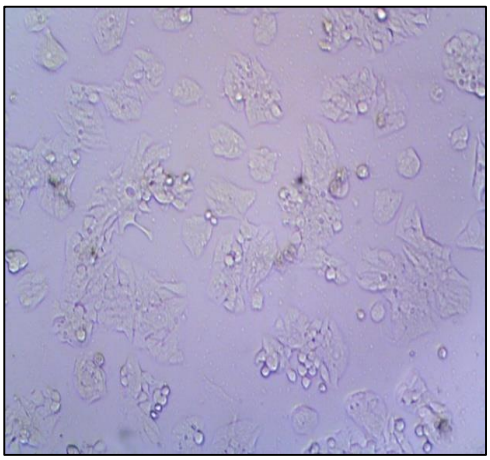
Figure 58: HT-29 Cell Line Inhibition By Compound PZ-6



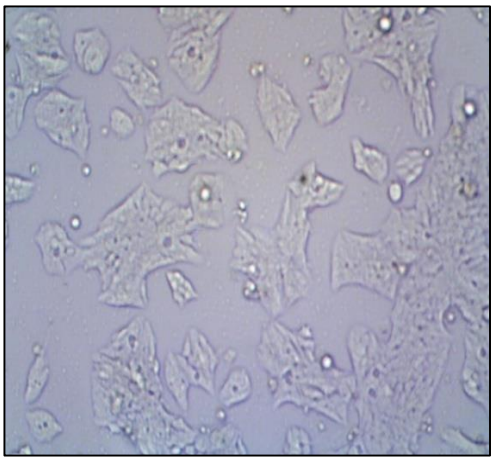
0.25 µM



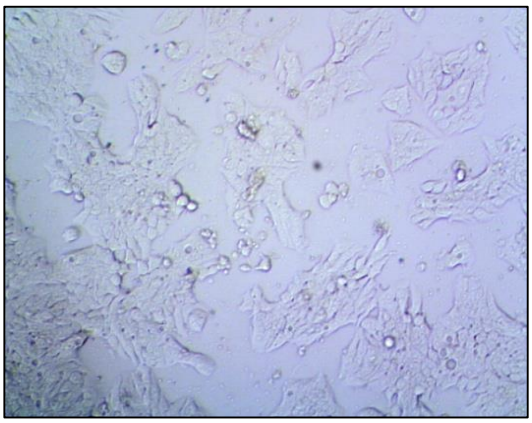
2.5 µM



25 µM

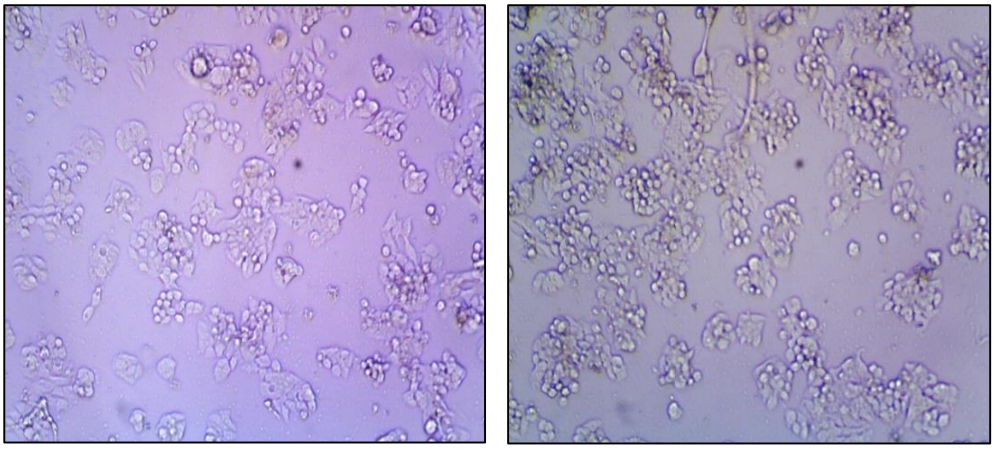


50 µM

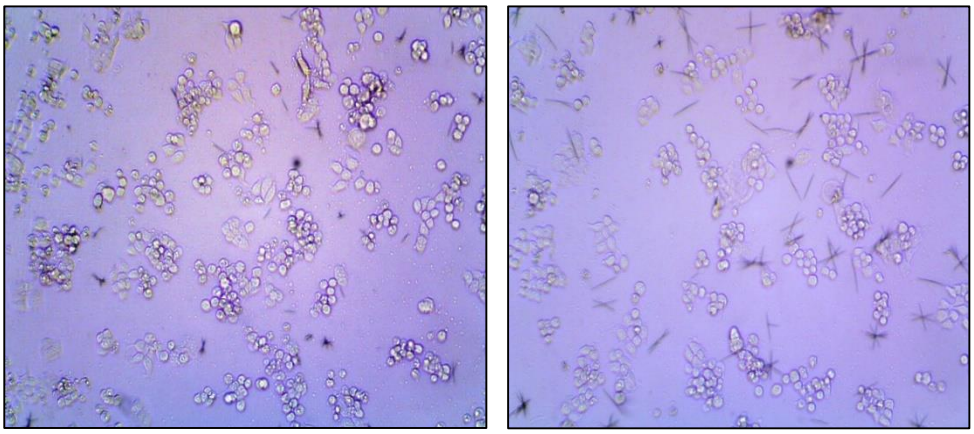


100 µM

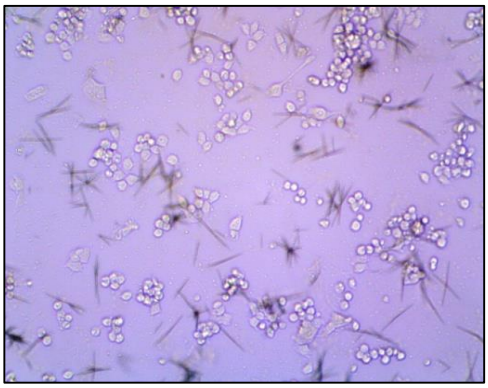
Figure 59: HT-29 Cell Line Inhibition By Compound PZ-5



0.25 µM **2.5 µM**



25 µM **50 µM**



100 µM

FIGURE 60: % CELL GROWTH OF COMPOUND PZ-3

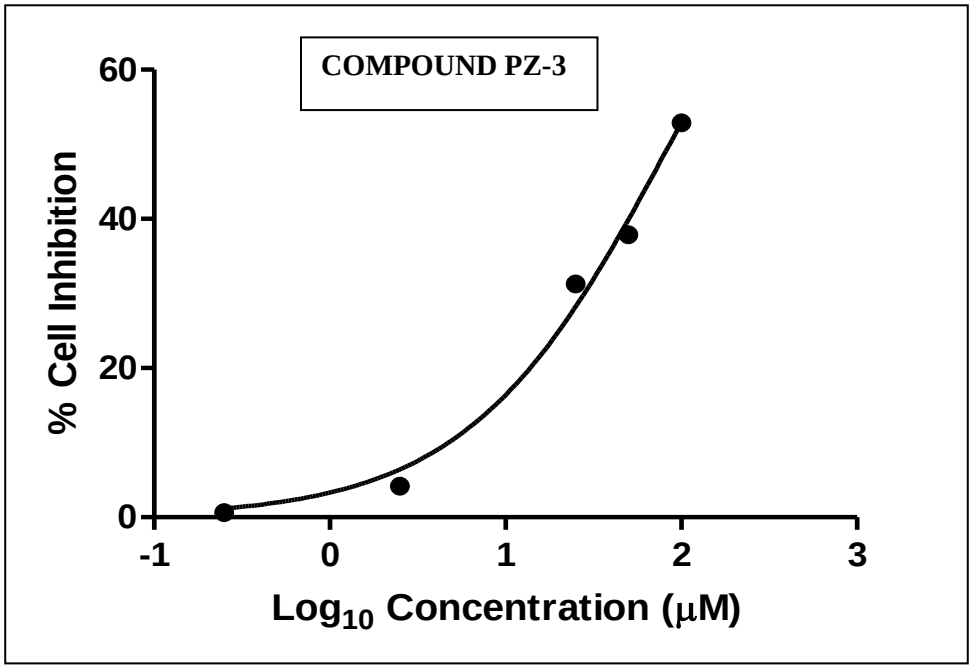
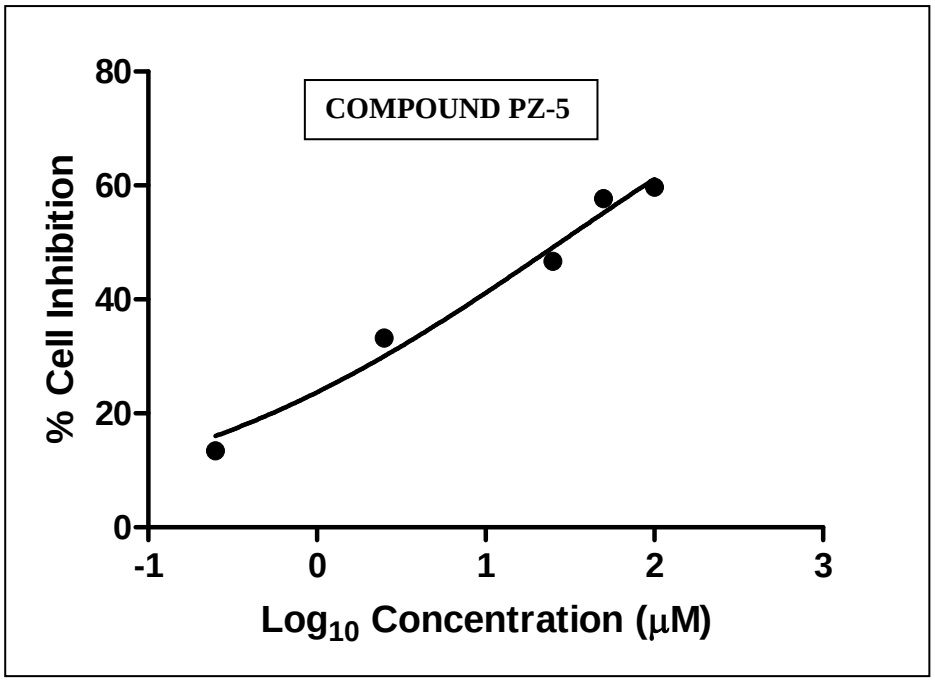


FIGURE 61: % CELL GROWTH OF COMPOUND PZ-5



ACUTE ORAL TOXICITY STUDY

Acute toxicity study was done according to OECD 423 guideline. No mortality was observed to a dose as 2000mg/kg p.o of (PZ-5) and the results showed that the compound could be used safely in the animals up to the dose of 2000mg/kg. Dose levels for the present study were chosen as 100mg/kg and 200mg/kg.

Table 24: Effect of Vascular Endothelial Growth Factor Receptor Inhibitor (PZ-5) on Acute toxicity in Female Wistar Rat

Sl no:	Response	Head		Body		Tail.	
		Before.	After.	Before.	After.	Before.	After.
1	Alertness	Normal	Normal	Normal	Normal	Normal	Normal
2	Grooming.	Absent	Absent	Absent	Absent	Absent	Absent
3	Touch response	Absent	Absent	Absent	Absent	Absent	Absent
4	Torch response	Normal	Normal	Normal	Normal	Normal	Normal
5	Pain response	Normal	Normal	Normal	Normal	Normal	Normal
6	Tremor.	Absent	Absent	Absent	Absent	Absent	Absent
7	Convulsion	Absent	Absent	Absent	Absent	Absent	Absent
8	Righting reflux	Normal	Normal	Normal	Normal	Normal	Normal
9	Gripping strength	Normal	Normal	Normal	Normal	Normal	Normal
10	Pinna reflux	Present	Present	Present	Present	Present	Present
11	Corneal reflux	Present	Present	Present	Present	Present	Present
12	Writhing	Absent	Absent	Absent	Absent	Absent	Absent
13	Pupils	Normal	Normal	Normal	Normal	Normal	Normal
14	Urination	Normal	Normal	Normal	Normal	Normal	Normal
15	Salivation	Normal	Normal	Normal	Normal	Normal	Normal
16	Skin colour	Normal	Normal	Normal	Normal	Normal	Normal
17	Lacrimation	Normal	Normal	Normal	Normal	Normal	Normal

EXPERIMENTAL DESIGN FOR DMH INDUCED COLON CANCER

In the present study, DMH induced colon carcinoma in Sprague Dawley rats was performed.1,2-Dimethyl hydrazine(DMH) is a potent colon specific carcinogen and an alkylating agent can cause colon cancer which mimics human colon cancer and is therefore, an ideal model for chemoprevention studies.

Body weight analysis

Body weight analysis was carried out on the basis of change in initial,intermediate and final body weight of control (only DMH) and treated animals. At the time of tumour development, animal body weight will be reduced. The intraperitoneal administration of DMH to the rats showed significant decrease in the body weight when compared to normal control rats. In case of treatment groups after the administration of VEGFR2 inhibitor (PZ-5) at different doses(100mg/kg and 200mg/kg) showed considerable prevention of weight loss when compared to DMH control rats.

Table 25: Body weight analysis

GROUPS (n =6)		Normal	Only DMH	DMH+STD (5-FU-20mg/kg)	DMH+PZ-5 Low Dose (100 mg/kg)	DMH+PZ-5 High Dose (200mg/kg)
Body weight (g)	Initial day	161.2±2.833	148.0±8.571^{a,ns}	142.5±7.671^{b,ns}	158.3±0.9888^{b,ns}	149.2±5.375^{b,ns}
	Inter mediate day	212.5±5.353	155.8±2.088 ^{a***}	204.3±4.264^{b***}	172.0±4.698^{b,ns}	190.8±9.167^{b***}
	Final day	272.2±9.167	208.5±4.326 ^{a***}	240.7±2.917^{b**}	234.7±4.055^{b*}	238.7±4.333^{b**}

a- only DMH compared with normal Group

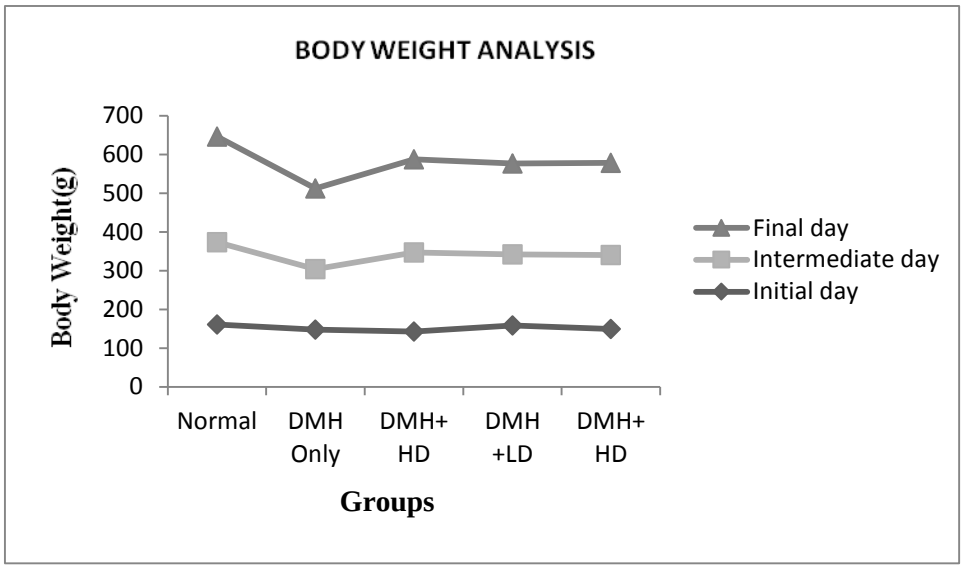
b- Group III, Group IV, Group V compared with only DMH.

Ns-non significant

Statistical comparison: One way ANOVA, followed by Tukey's comparison was performed. All values are expressed as mean± SEM. Only DMH group was compared with Control group. Group III (DMH+STD), group IV (DMH+ PZ-5 Low dose), group V

(DMH+PZ-5 High dose), was compared with only DMH. **(P<0.05-*, P<0.01-**, P<0.001-***)**

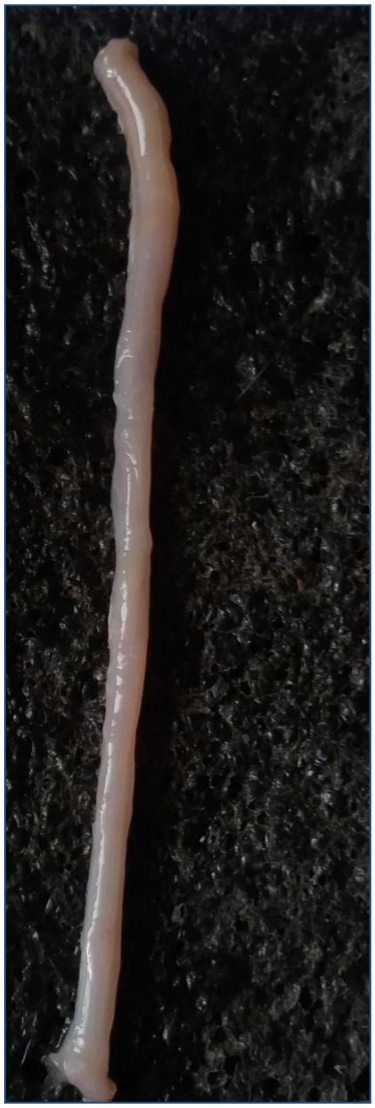
Figure 62: Body weight Analysis



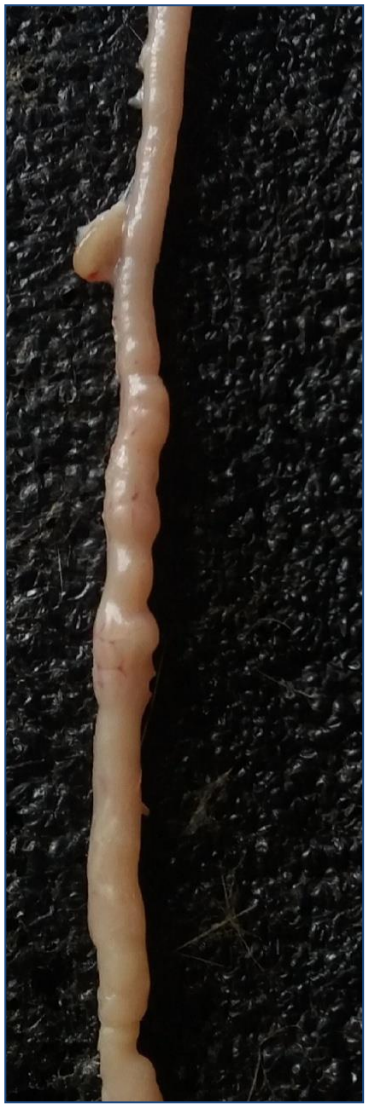
TUMOUR GROWTH

The negative control (only DMH) group showed a significant tumor growth compared with normal due to tumour induction.The treatment with VEGFR2 inhibitor (PZ-5-100mg/kg and 200mg/kg i.p) showed considerable prevention of of tumor growth as compared to DMH control rats and is given in figure 50.

Figure 63: Tumour Growth



Group I- Normal



Group II – Only DMH

Group III
DMH + STD
(5-FU-20mg/kg)

Group IV
DMH + PZ-5
(Low Dose-100mg/kg)

Group V
DMH + PZ-5
(High Dose -200mg/kg)

ESTIMATION OF TUMEROGENESIS

Estimation of tumerogenesis was carried out on the basis of changes in tumor burden and tumor weight is given in table 27. In this study, only DMH Group showed increase in tumour burden and tumour weight. The treatment with pyridazine derivative (PZ-5-100mg/kg and 200mg/kg i.p) shows moderate decrease in tumour burden and tumour weight and is shown in figure 63 & 64.

Table 26: Estimation of tumour burden and tumor weight

Groups (n =6)	Normal	Only DMH	DMH +STD (5-FU,20mg/kg)	DMH+ PZ-5 Low Dose (100 mg/kg)	DMH + PZ-5 High Dose (200mg/kg)
Tumour Burden (No.of polyps)	0	$11.50\pm0.56^{a***}$	$5.167\pm0.47^{b***}$	$8.833\pm0.65^{b**}$	$7.333\pm0.49^{b***}$
Tumour Weight (g)	1.120±0.03	$1.43\pm0.02^{a***}$	$1.113\pm0.04^{b***}$	$1.275\pm0.03^{b*}$	$1.181\pm0.05^{b***}$
Tumour (ACF) incidence(%)	—	100%	100%	100%	100%

a- only DMH compared with normal Group

b– Denotes when compared with only DMH.

One way ANOVA, followed by Dunnet's comparison was performed. Group III (DMH +STD), group IV (DMH + PZ-5 Low dose), group V (DMH + PZ-5 High dose), was compared with only DMH. (P<0.05-*, P<0.01-**, P<0.001- ***)

Figure 64: Estimation of tumour burden

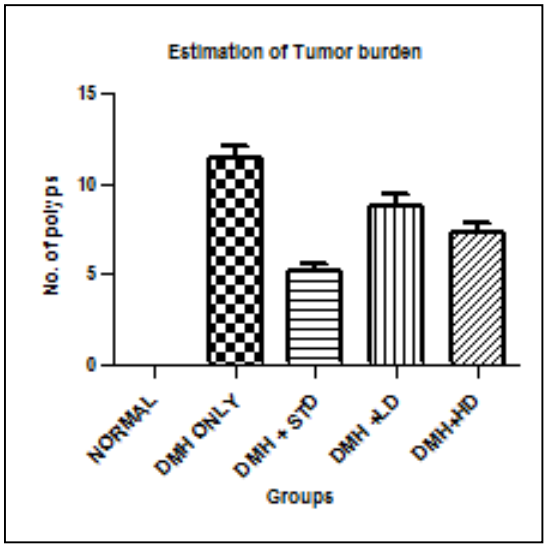
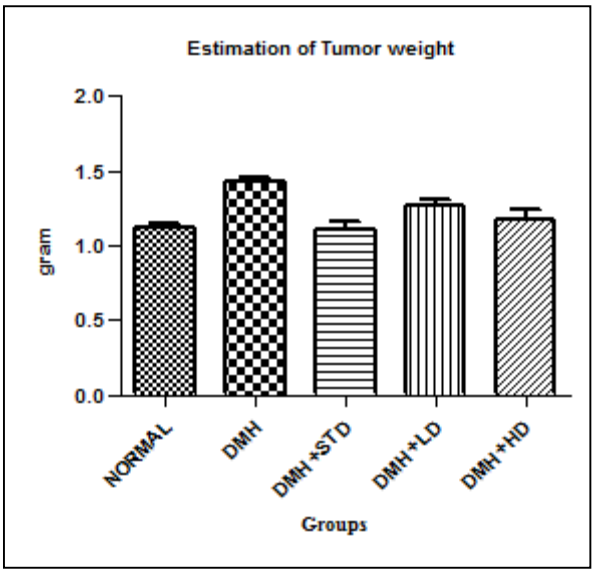


Figure 65: Estimation of tumour weight



ESTIMATION OF HAEMATOLOGICAL PARAMETERS

In DMH control rats, a significant decrease in the RBC and Hb levels were shown as compared to normal control rats, but the WBC level was increased. In the PZ-5 treated groups, the levels of RBC and Hb were increased and there is a significant reduction in the WBC level**.**

Data is expressed as Mean ± SEM(n=6, animals in each groups)

Table 27: Estimation of Haematological Parameters

Groups (n =6)	Normal	Only DMH	DMH +STD (5-FU, 20mg/kg)	DMH+ PZ-5 Low Dose (100 mg/kg)	DMH + PZ-5 High Dose (200mg/kg)
RBC ($1x10^{6}$ / L)	$7.823\pm0.1299^{a***}$	$6.148\pm0.04799^{b***}$	$7.633\pm0.1275^{b***}$	$7.008\pm0.2298^{b**}$	$7.743\pm0.08811^{b***}$
WBC ($1x10^{3}$/ L)	$10.08\pm0.4223^{a***}$	$14.35\pm0.1310^{b***}$	$10.08\pm0.5469^{b***}$	$12.95\pm 0.1302^{b*}$	$10.61\pm 0.3480^{b***}$
Hb (g/dl)	$14.42\pm0.4643^{a***}$	$9.850\pm0.1803^{b***}$	$14.00\pm0.4648^{b***}$	$13.15\pm0.6464^{b***}$	$13.72\pm0.4792^{b***}$

a-only DMH compared with normal Group,
b- Group III, Group IV, Group V compared with only DMH.

Statistical comparison: One way ANOVA, followed by Dunnet's comparison was performed. Only DMH group was compared with Normal group. Group III (DMHV+STD),

group IV (DMH +PZ-5 Low dose), group V (DMH+ PZ-5 High dose), was compared with only DMH.

(P<0.05-*, P<0.01-, P<0.001- ***)**

Figure 66: Enumeration of RBC

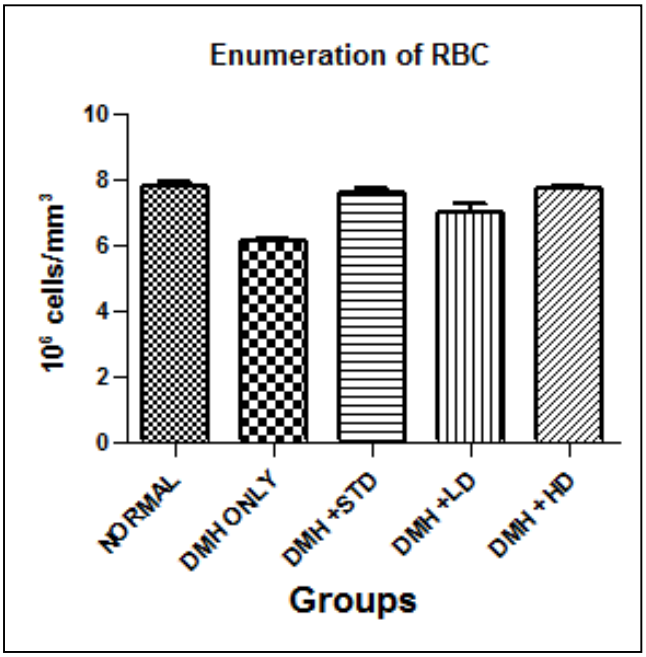


Figure 67: Enumeration of WBC

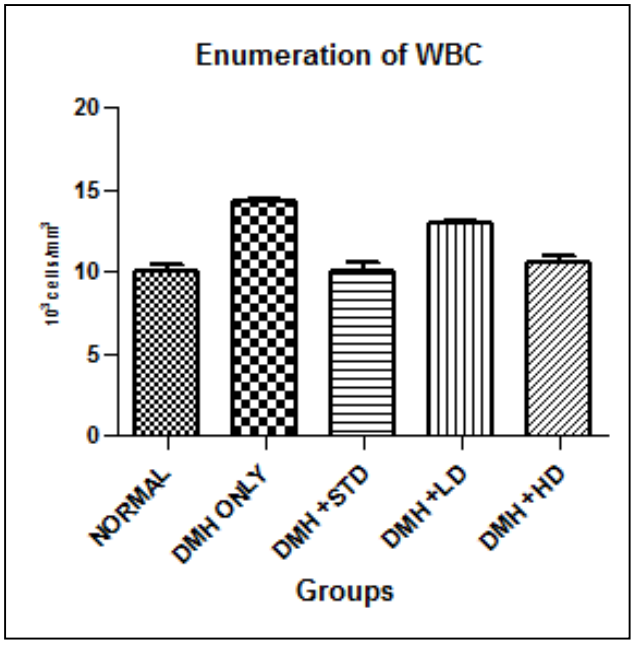
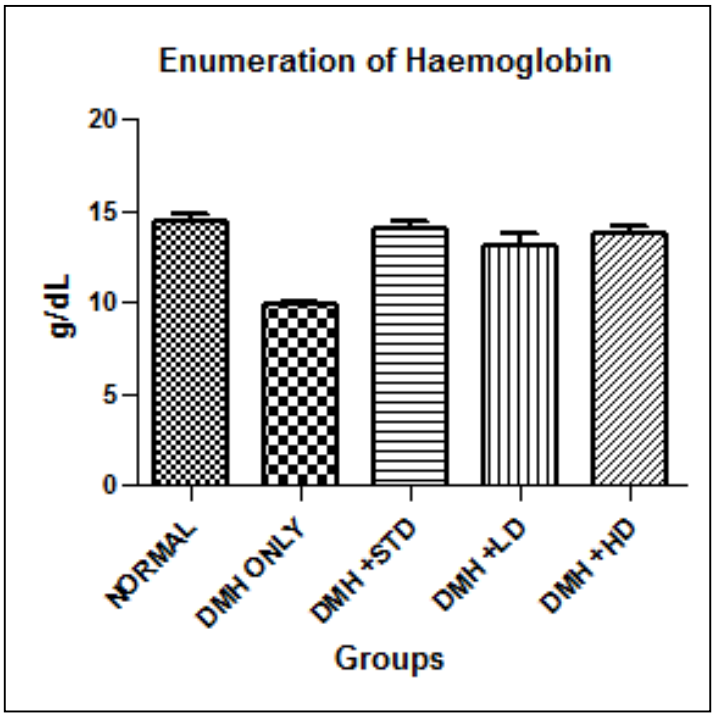


Figure 68: Estimation of Haemoglobin



ESTIMATION OF SERUM BIOCHEMICAL PARAMETERS

Serum biochemical levels estimation is one the major diagnosing tool to confirm liver diseases.The parameters include ALP, SGOT, SGPT, total protein and creatinine and these parameters are considered as the tumour marker in this study.A significant increased serum biochemical parameter levels were observed in the DMH control rats when compared to normal control rats. Rats treated with PZ-5 showed significantly lower biochemical levels when compared to DMH control.

Data is expressed as Mean ±SEM .(n=6, animals in each groups)

Table 28: Estimation Of Serum Biochemical Parameters

Groups (n =6)	Only DMH	Normal	DMH +STD (5-FU, 20mg/kg)	DMH+ PZ-5 Low Dose (100 mg/kg)	DMH + PZ-5 High Dose (200mg/kg)
ALP (U/L)	174.3±2.860	85.67±2.044^{a***}	105.7±2.578^{b***}	144.0±6.608 ^{b***}	112.8±1.641 ^{b***}
SGOT (U/L)	99.33±2.565	46.17±1.249 ^{a***}	61.67±2.319^{b***}	82.83±4.735 ^{b***}	62.17±2.120 ^{b***}
SGPT (U/L)	93.17±1.376	26.67±1.706 ^{a***}	38.00±1.125 ^{b***}	56.33±5.077 ^{b***}	41.00±1.713 ^{b***}
Creatinine (mg/dl)	0.8667±0.06667	0.4667±0.0500 ^{a***}	0.4833±0.03073 ^{b***}	0.6000±0.03651 ^{b**}	0.5000±0.04472 ^{b***}
Total protein (mg/dl)	7.833±0.1382	4.117±0.08724 ^{a***}	4.700±0.1183 ^{b***}	7.050±0.2405 ^{b*}	6.850±0.2553 ^{b**}

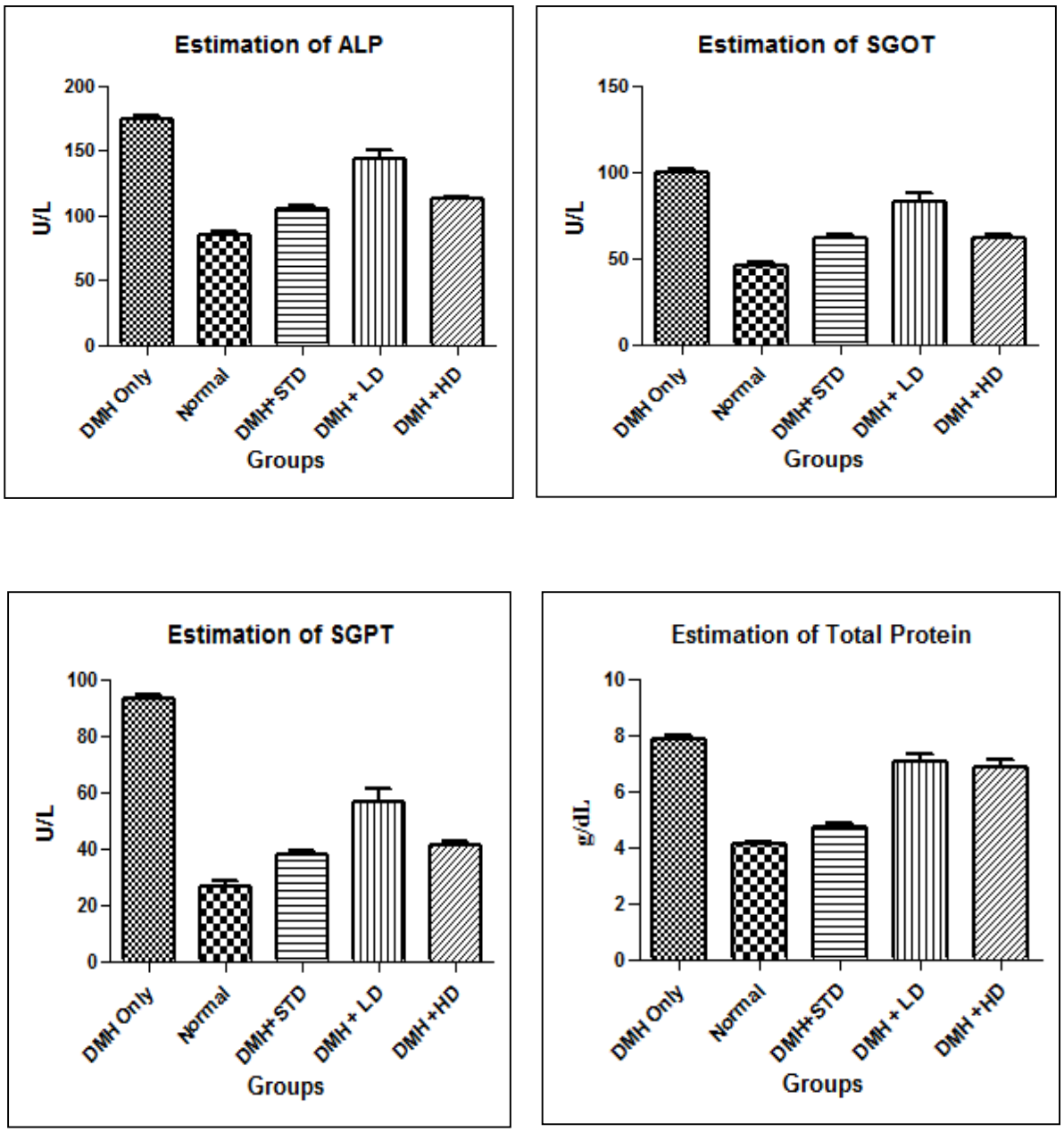
a-only DMH compared with normal Group

b- Group III, Group IV, Group V compared with only DMH.

ns -nonsignificant

Statistical comparison: One way ANOVA, followed by Dunnet's comparison was performed. Only DMH group was compared with Normal group. Group III (DMH+STD), group IV (DMH+PZ-5 Low dose), group V (DMH+ PZ-5 High dose), was compared with only DMH. (**P<0.05-*, P<0.01-**, P<0.001- ***)**

Figure 69:Estimation of Serum Biochemical Parameters



ESTIMATION OF INVIVO ANTIOXIDANT ACTIVITY

Living organisms have developed several effective mechanisms to get protection from the reactive oxygen species. *In vivo* antioxidant system can be divided into enzymatic and non

enzymatic. The enzymatic defence system includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPx) and non enzymatic defence system includes reduced glutathione (GSH) and invivo antioxidant levels in colon was shown in figure.

Super Oxide Dismutase

The cells containing this enzyme scavenges superoxide ion and prevents its accumulation so that cells are protected from oxidative stress. SOD catalyses this dismutation of superoxide in which one O_2* is oxidized to O_2 while the other is reduced to H_2O_2. The formed H_2O_2 is able to damage the cellular components and are instantly removed by enzymes like catalase and GPx. A significant decrease in SOD levels was observed in DMH control rats when compared to normal control rats. The levels get increased in the rats treated with compound PZ-5 and 5-Fluorouracil when compared to SOD levels in DMH control rats.

Catalase

Oxidative stress has a major role in carcinogenesis. The catalase enzyme is an endogenous antioxidant enzyme that neutralizes reactive oxygen species by converting H_2O_2 into H_2O and O_2. Among the other antioxidant enzymes, including superoxide dismutase and glutathione peroxidase, catalase is an elementary defense against oxidative stress. In DMH control rats, a significant decreased level of catalase was observed as compared to normal control rats. The compound PZ-5 and 5-Fluorouracil treated groups showed significant increase in catalase levels when compared to DMH control rats.

Reduced Glutathione

It prevents free radical induced oxidation of SH groups of various proteins to disulfide derivatives. It also protects haemoglobin from getting oxidized by H_2O_2. A significant decrease in the GSH levels was observed in the DMH control rats when compared to normal control rats. Rats treated with PZ-5 and 5-Fluorouracil showed significantly higher GSH levels when compared to DMH control rats.

Glutathione Peroxidase

The biochemical function of GPx involves the reduction of lipid hydroperoxides to their alcohols and to reduce free hydrogen peroxide to water. Thus, it protects the cell from oxidative damage. The DMH control rats showed significant lower GPx levels when

compared to the normal control rats. The compound PZ-5 and 5-Fluorouracil treated rats showed significant higher GPx levels when compared to DMH control rats.

Lipid Peroxidation

Lipid peroxidation indicates the oxidative degradation of lipids in which the free radicals steals electrons from the lipids in the cell membrane and leads to cell damage. This process proceeds by a free radical chain reaction mechanism. Radical reaction consists of initiation, propagation and termination processes. A significant increased level of LPx was observed in the DMH control rats when compared to normal control rats. The compound PZ-5 and 5-Fluorouracil treated rats showed significant decreased LPx levels when compared to DMH control rats.

Mitochondrial TCA-cycle enzymes

As mitochondria plays a critical role in bioenergetics, anabolic and cell death pathways which range from high tissue-specific conditions to generalized whole-body disorders including cancer. Several common features of established tumour cells can directly or indirectly result from mitochondrial deregulation. Mitochondria may be implicated in early tumerogenesis.

Data is expressed as Mean ±SEM .(n=6, animals in each groups)

Table 29: *In vivo* antioxidant levels in Colon

Groups	Antioxidant enzymes(Colon)				
	SOD(unit/min/mg protein)	CATALASE (µmol of H_2O_2 consumed/min/mg protein)	GSH (Glutathione µg/mg)	GP_x(nmol of glutathione oxidized/min/mg protein)	LPO (nmol of MDA/mg protein)
Normal	5.533±0.07149	41.83±0.7923	19.74±0.3712	5.280±0.2251	7.607±0.3664
Only DMH	2.150±0.1118^{a***}	22.67±1.054 ^{a***}	11.21±0.1727^{a***}	2.773±0.1098^{a***}	25.77±0.3831 ^{a***}
DMH +STD (5-FU, 20mg/kg)	5.133±0.1706^{b***}	39.17±0.7032 ^{b***}	18.94±0.2929^{b***}	4.763±0.1173^{b***}	8.998±0.2671 ^{b***}

DMH+PZ-5 Low Dose (100 mg/kg)	3.000±0.1317^{b***}	34.83±1.493 ^{b***}	15.30±0.5102^{b**} *	3.642±0.1259^{b***}	12.64±0.5315 ^{b***}
DMH+PZ-5 High Dose (200 mg/kg)	4.983±0.09098^{b**} **	37.83±0.9458 ^{b***}	17.80±0.2452^{b**} *	4.607±0.1028^{b***}	9.632±0.4543 ^{b***}

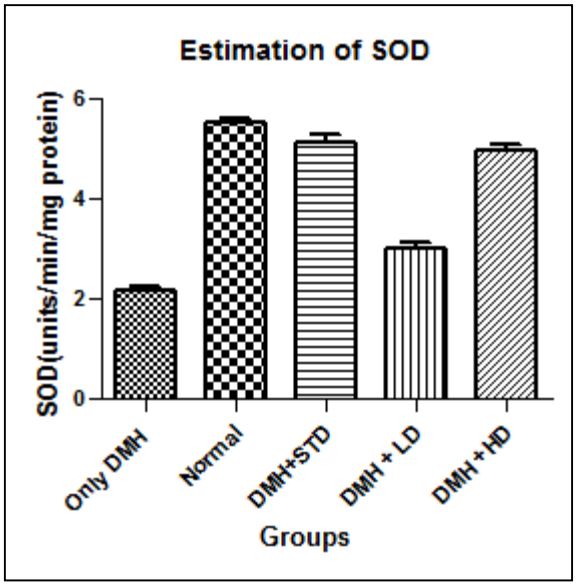
a- only DMH compared with normal Group

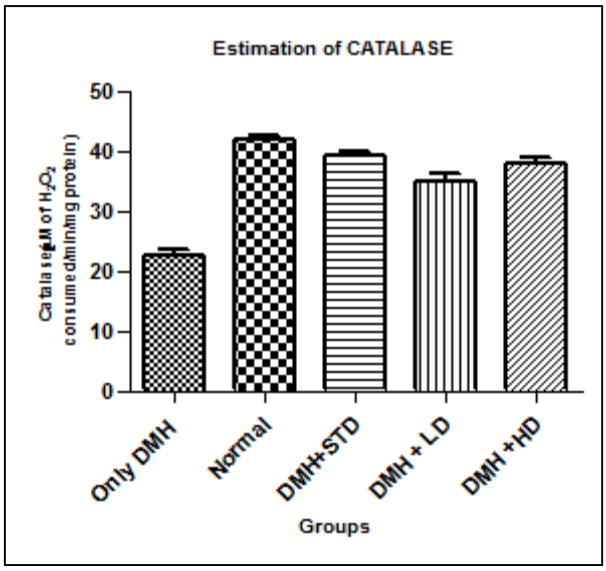
b- Group III, Group IV, Group V compared with only DMH.

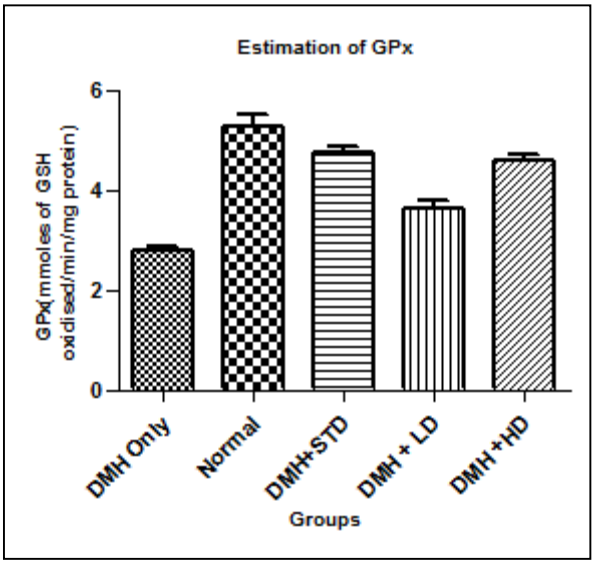
ns –non significant

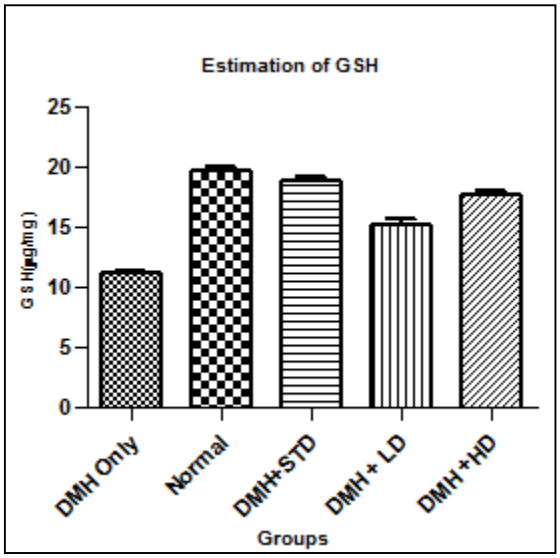
Statistical comparison: One way ANOVA, followed by Dunnet's comparison was performed. Only DMH group was compared with Normal group. Group III (DMH+STD), group IV (DMH+PZ-5 Low dose), group V (DMH + PZ-5 High dose), was compared with only DMH. (**P<0.05-*, P<0.01-**, P<0.001- ***)**

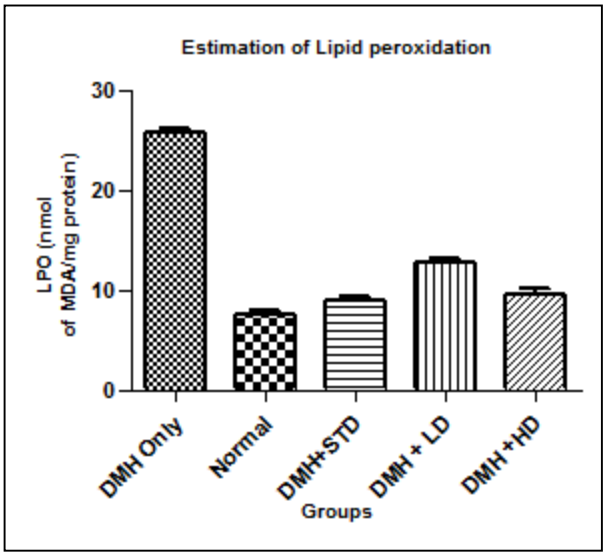
Figure 70: *In vivo* antioxidant levels in Colon




Estimation of CATALASE
Catalase(μM of H_2O_2 consumed/min/mg protein)
50
40
30
20
10
0
Only DMH
Normal
DMH+STD
DMH + LD
DMH +HD
Groups


Estimation of GPx
GPx(mmoles of GSH oxidised/min/mg protein)
6
4
2
0
DMH Only
Normal
DMH+STD
DMH + LD
DMH +HD
Groups





Estimation of Total protein- (Liver)

Table 43: shows total protein levels in Liver

Data is expressed as Mean ±SEM .(n=6, animals in each groups)

Table 30: Estimation of Total protein

GROUPS (n =6)	DMH Only	Normal	DMH +STD (5-FU,20mg/kg)	DMH + PZ-5 LOW DOSE (100 mg/kg)	DMH+ PZ-5 HIGH DOSE (200 mg/kg)
Total protein (mg/100mg tissue)	2.800±0.2280	5.317±0.4658^{a***}	4.800±0.2236^{b***}	4.100±0.1592^{b**}	4.583±0.1138^{b***}

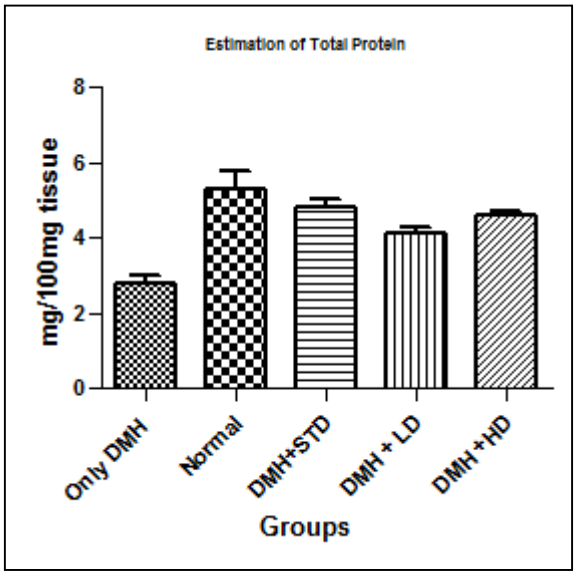
a- only DMH compared with Normal Group

b- Group III, Group IV, Group V compared with only DMH

Statistical comparison: One way ANOVA, followed by Dunnet's comparison was performed. Only DMH group was compared with Control group. Group III (DMH+STD), group IV (DMH+ PZ-5 Low dose), group V (DMH+ PZ-5 High dose), was compared with only DMH.

(P<0.05-*, P<0.01-, P<0.001- ***)**

Figure 71: Estimation of Total protein



ESTIMATION OF TCA CYCLE ENZYME

Estimation of succinate dehydrogenase

Succinate dehydrogenase

The Succinate Dehydrogenase level was found to be significantly decreased in DMH control rats when compared to normal control rats. Rats treated with compound PZ-5 and 5-FU showed significantly higher Succinate Dehydrogenase levels when compared to DMH control rats

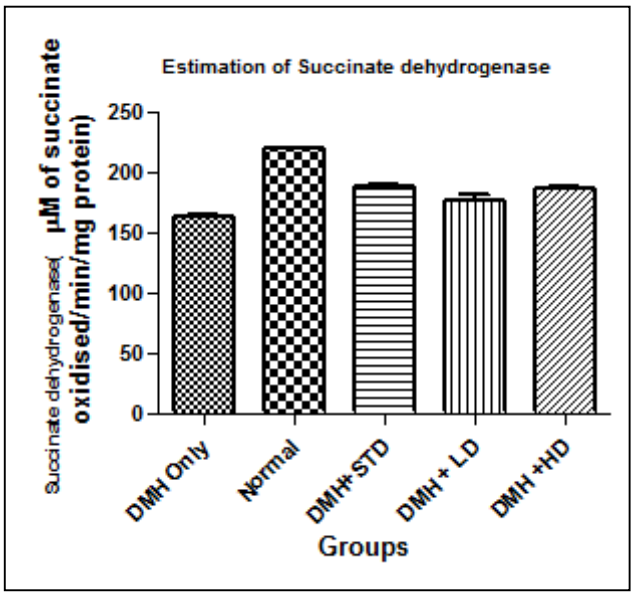
Table 31:: shows Succinate dehydrogenase levels in Liver

GROUPS (n =6)	NORMAL	ONLY DMH	DMH+STD	DMH +PZ-5 Low Dose (100 mg/kg)	DMH+ PZ-5 High Dose (200mg/kg)
Succinate Dehydrogenase (µmol of succinate oxidised/min/mg protein)	218.8±0.9458	163.5±1.727[a**] *	188.3±1.054[b**] *	175.7±2.352[b] *	186.5±1.258[b**] *

All values are expressed as mean± S.E.M; (n=6) animals in each group.

Statistical comparison: One way ANOVA, followed by Dunnet's comparison was performed. Only DMH group was compared with Control group. Group III (DMH+STD), group IV (DMH+ PZ-5 Low dose), group V (DMH+ PZ-5 High dose), was compared with only DMH.

Figure 72:Estimation of succinate dehydrogenase

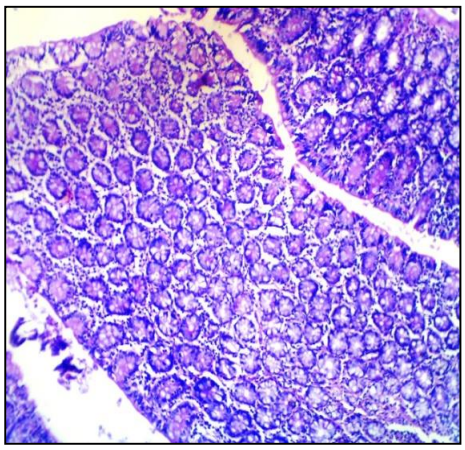


HISTOPATHOLOGICAL ANALYSIS

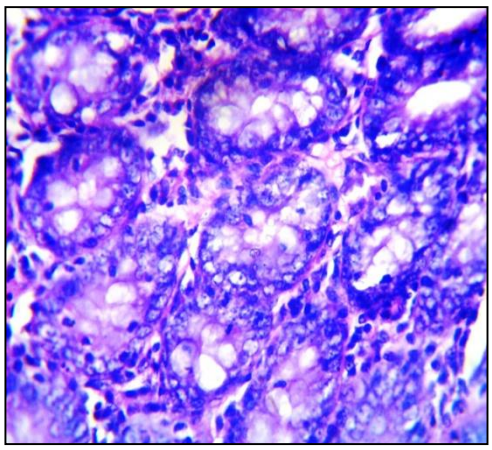
Histopathological analysis of tumours of various groups revealed that only DMH group shows high amount of infiltration whereas the level of infiltration for standard was less when compared with that of test compound PZ-5.

Figure 73: Histopathology of Colon tumours

GROUP –I Normal

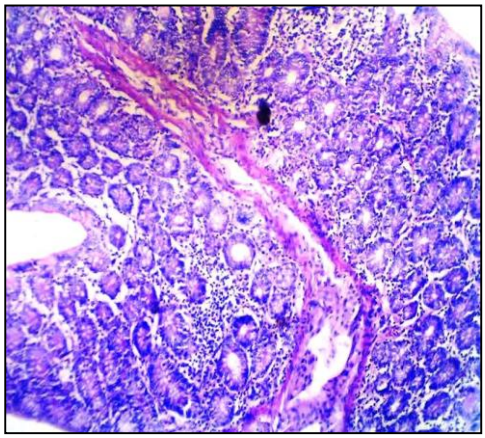


10x shows Colonic mucosa

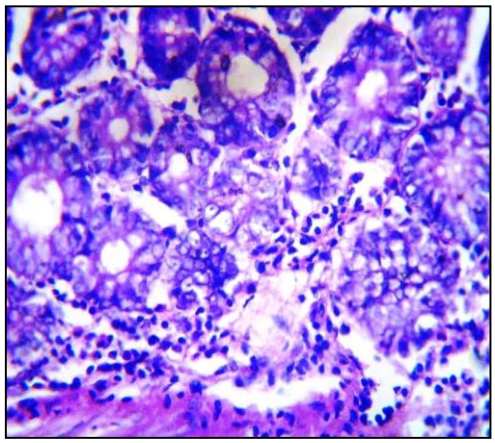


40x shows Colonic mucosa

GROUP –II Only DMH

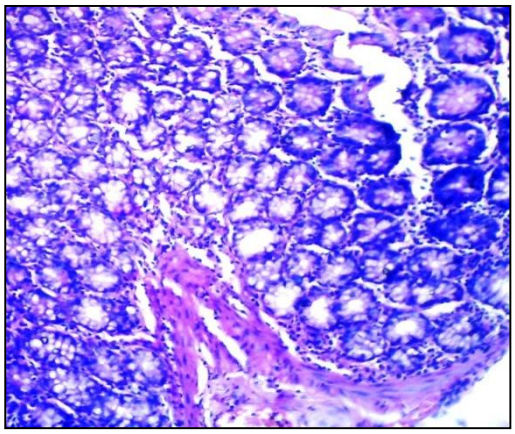


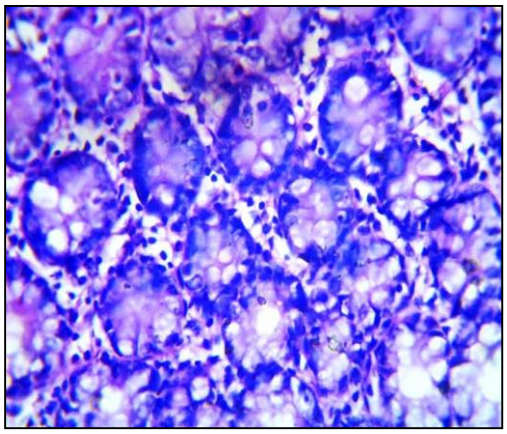
10x shows Colonic mucosa with dysplastic changes.



40x shows mild dysplastic cells with occasional mitosis

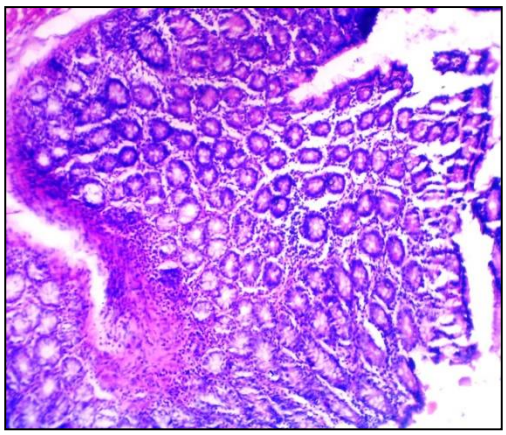
GROUP – III DMH+ STD

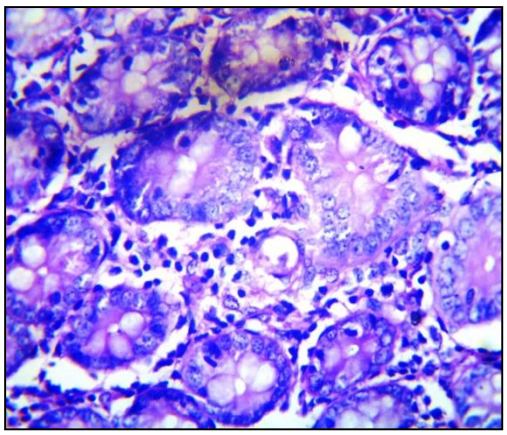




10x shows Colonic mucosa with mild inflammation.40x shows normal mucosa with inflammatory infiltrates in the lamina propria

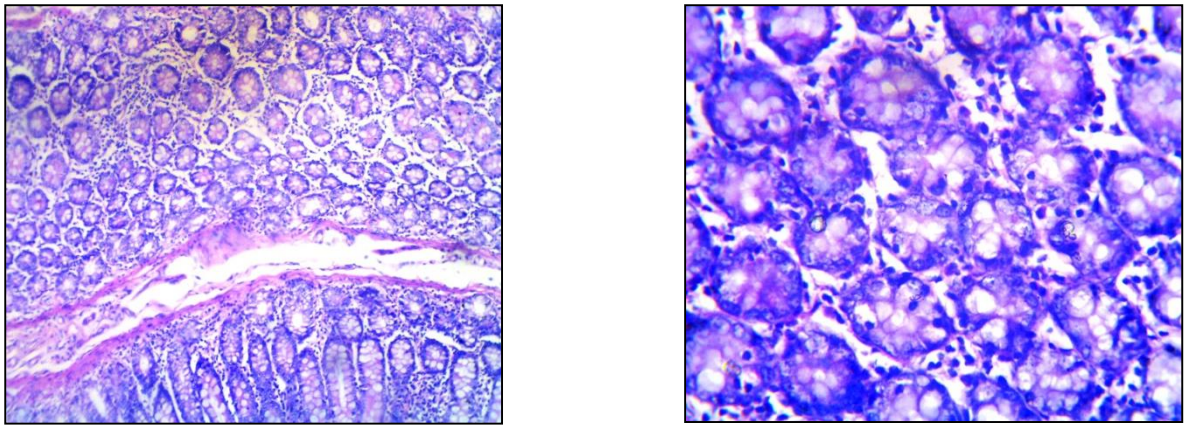
GROUP – IV DMH+ Test compound PZ-5 Low Dose 100 mg/kg





10x shows normal Colonic mucosa with muscular layer and submucosa.40x shows lamina propria shows mild inflammation

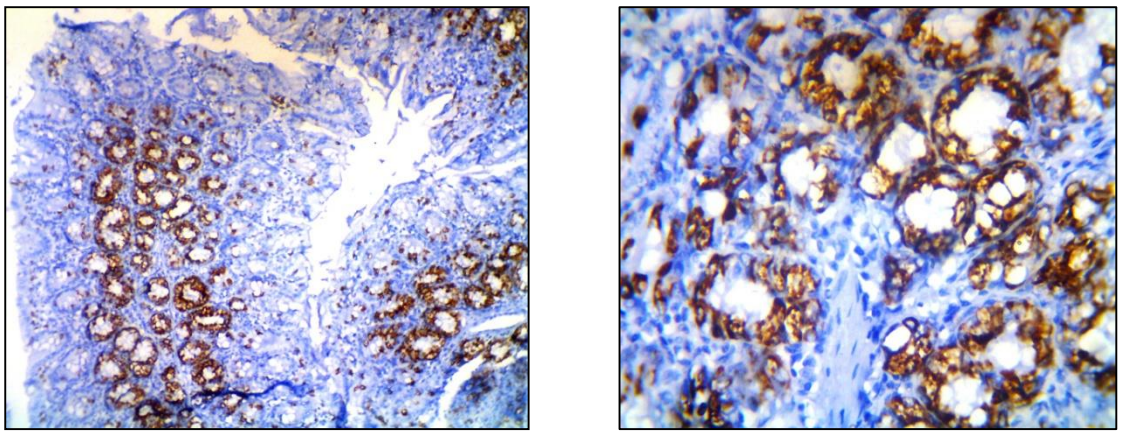
GROUP –V DMH + Test Compound PZ-5 High Dose 200 mg/kg



10x shows normal mucosa,sub mucosa_and muscular layer. 40x shows Colonic mucosa with infiltrates

Figure 74: Immunohistochemical Reaction

GROUP – II Only DMH

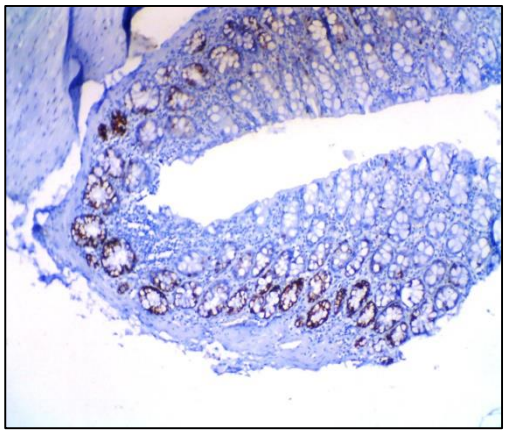


10x **40x**

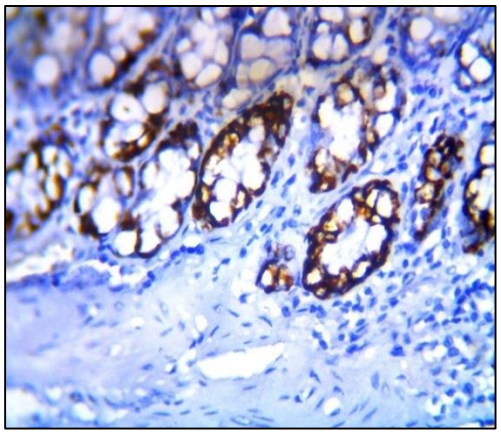
COX -2: Cytoplasmic postivity in inflammatory cells (2+)

GROUP – III DMH+ Std

(5-FU-20mg/kg)



10x

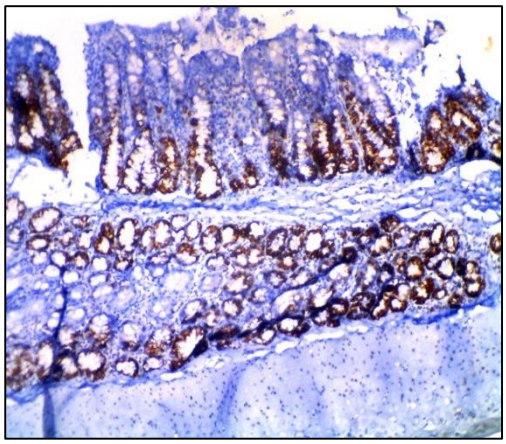


40x

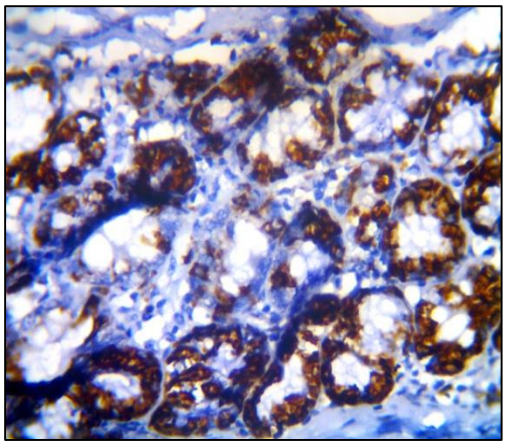
COX -2: Cytoplasmic postivity in inflammatory cells (2+)

GROUP – IV (DMH+PZ-5)

Low Dose 100 mg/kg



10x

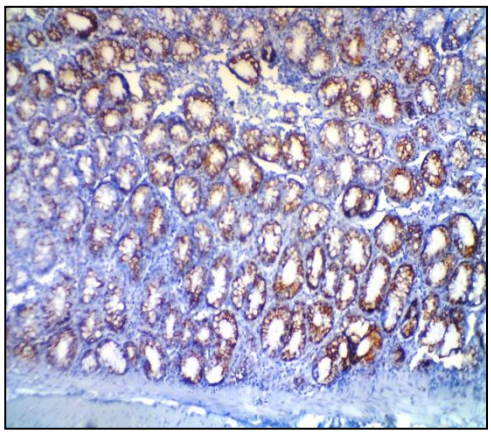


40x

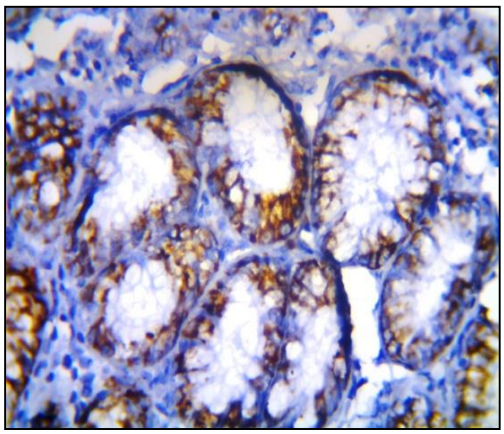
COX -2: Cytoplasmic postivity in inflammatory cells (2+)

GROUP – V (DMH+PZ-5)

High Dose 200 mg/kg



10x



40x

COX -2: Cytoplasmic postivity in inflammatory cells (2+)

7. DISCUSSION

Cancer has reached epidemic proportions globally and accounts for 12 million cases worldwide and 7.9 million deaths. The current challenges of anticancer drug development include the significant time and cost involvement, and the low success rates. These issues lead to increasing efforts of the pharmaceutical industry toward increasing the effectiveness of the drug discovery and development process to minimize failure of drug candidates at later stages of development. It also includes development of high throughput preclinical screening methods (computational molecular modeling techniques) and biological assays with greater specificity and predictability. The advanced search for newer avenues in the treatment of cancer has led research into the elucidation of signaling pathways.[29]

The recent growth in molecular sciences and the advances in genomics and proteomics paradigms the anticancer drug discovery towards molecularly targeted therapeutics.

The target selection is a crucial step in modelling, among these targets, angiogenesis is one of the critical processes that affect growth and development of cancerous cells. The Vascular endothelial growth factor receptor(VEGF) are selected as target protein,which is one of the most potent angiogenic factors. Vascular endothelial growth factor (VEGF) signaling through VEGF receptor 2(VEGFR 2) has been implicated to play a key role in the regulation of tumor angiogenesis. VEGF is a homodimeric cytokine that is expressed in atleast four splice-variant forms of 121-206 residues. Over expressed VEGF correlates with poor prognosis and clinical stage in solid tumor patients. Therefore,VEGF/VEGFR2 signaling is an attractive target for the treatment of cancer.[24]

During the course of pharmaceutical development of novel VEGFR2 inhibitors, pharmacophore and docking based in silico studies are efficiently used to improve the discovery of lead identification and optimization, which is followed by the synthesis of lead compound derivatives and their biological evaluation. In silico Molecular modelling studies will lead to invention of a potent molecule in less time with

reduction in the usage of chemicals and animals, it will be a process of In-cerebro-In-silico and chemico approach of drug designing.

Pyrazolopyridazine have garnered attention due to its wide variety of biological activities which are structural analogues of many purine nucleosides moreover it was identified through high throughput screening as a potent inhibitor of VEGFR2 and has remarkable anticancer activity. It is also found to be cytotoxic against a wide variety of cancer cell lines which prompted us to select the nucleus.

Lead Optimisation[11]

Lead optimisation was performed for the designed 54 Pyrazolo benzpyridazine derivatives by Molispiration software which showed that all compounds followed Lipinski's rule of five. Compounds that satisfy these rules are considered as drug like and were analysed for their ADMET toxicity. Statistics reports show that a lot of drug candidates are failed during clinical tests because of the problems related to ADME, and nowadays researchers consider ADME properties as important condition to choose compounds as drug candidates. Numerous *in vitro* methods have been used in the drug selection process for assessing the intestinal absorption of drug candidates. Among them, Caco-2 cell model has been recommended as a reliable *in vitro* model for the prediction of oral drug absorption.

- Predicting human intestinal absorption of drugs is very important for identify potential drug candidate. In PreADMET can predict percent human intestinal absorption (%HIA). Human intestinal absorption data are the sum of bioavailability and absorption evaluated from ratio of excretion or cumulative excretion in urine, bile and feces.
- Blood-Brain Barrier (BBB) penetration is represented as BB = [Brain]/[Blood], where [Brain] and [Blood] are the steady-state concentration of radiolabeled compounds in brain and peripheral blood. Predicting BBB penetration means predicting whether compounds pass across the blood-brain barrier. This is crucial in pharmaceutical sphere because CNS-active compounds must pass across it and

CNS-inactive compounds mustn't pass across it in order to avoid of CNS side effects.

From all the above parameters studied, it was found that compound PZ-5 is best among all the compounds designed.

Analysis of the many available crystal structures of kinase/ inhibitor complexes reveals the existence of conserved interactions that appear to be determinant for the recognition of small heterocyclic molecules by the ATP (cofactor) binding site of this class of enzymes. Prominent among these are hydrogen bond interactions that inhibitors make with the backbone of the amino acid stretch that connects the kinase N- and C-terminal domains, the so called 'hinge' loop. In particular, for VEGFR2, at least one hydrogen bond with hinge residue Cys 919 is observed in all reported crystal structures. These hydrogen bonds position the inhibitors in an orientation that allow them to make multiple favourable contacts with the side chains of hydrophobic residues that normally form the environment of the adenine ring of ATP in the pocket. In the case of VEGFR2, these residues include Leu1035A, Ala866A,Val916A and Lys868A.Our strategy to find new VEGFR2 inhibitors is based on the design of molecular scaffolds targeting ATP interactions so we used an X-ray crystal structure of VEGFR2 (PDB ID: 3VO3) to design pyrazolo benzpyridazine ligands.

A library of 54 ligands were virtually designed and docked to the VEGF protein. Based on the docking scores, amino acid interactions, drug likeliness and ADME prediction, feasibility of synthesis, 6 compounds were selected for synthesis.

Synthesis of the compounds involves the following steps:

Step 1: Synthesis of Ethyl 3 –oxo-2-(2-substituted phenylhydrazinylidene) butanoate.

Step 2: Synthesis of 3-Acetyl benzpyridazine-4(1*H*)-one derivatives.

Step 3: Synthesis of Pyrazolo benzpyridazine derivatives

Physical parameters like percentage yield, molecular weight, molecular formula, melting point, logP and $R_f$value of the synthesized compounds were determined. The final

compounds were in moderate to higher yields. The percentage yield was found to be in the range of 65 – 90 %.

The structures of the synthesized compounds were confirmed by IR, NMR, and mass spectral analysis.IR Spectrum of the synthesized compounds showed the characteristic absorption band at 2911-2926.93 cm^{-1},3162.69-3484.26 cm^{-1}, 1588.59-1629.55 cm^{-1},1045.71-1249.16 cm^{-1},1405.37-1667 cm^{-1} ,2926-3162.69 cm^{-1} arising from stretching vibration of bands (C-H, N-H, N=N, C-N, C=N, Aromatic C-H respectively), 1530.24 cm^{-1},1145.03 cm^{-1} ,3228.74-3302.02 cm^{-1} ,1096 cm^{-1} due to NO_2 stretching, C-F stretching, C-SO_2NH_2 stretching and C-Cl stretching respectively which confirms the chemical structure of the compounds synthesized.PMR spectra of the synthesized compounds derivatives shows peaks ranging from 10.69-11.61ppm,7-7.99 and 2.1-2.3ppm which is characteristic of H in NH, Aromatic H and H in CH_3 respectively. Thus the proton magnetic spectrum of the compound was in full agreement with its molecular formula, with regard to proton count and the chemical shift also.The Mass Spectral analysis of the synthesized compounds PZ-1 and PZ-5 were performed, and the mass spectrum of the compound was in agreement with its molecular weight.

DPPH and ABTS *in vitro* antioxidant activity were performed in synthesised compounds and they showed good antioxidant activity. The effect of antioxidants on DPPH and ABTS was thought to be due to their hydrogen donating ability. The free radical scavenging activity of the extract was estimated by comparing the % inhibition of synthesized compounds(PZ-1 to PZ-6) with standard ascorbic acid. The activity was found to be increased in a dose dependent manner.

In order to confirm the activity of the synthesized compounds against colon cancer, the compounds were tested for their cytotoxicity against colon cancer cell lines (HT-29). All the compounds tested were found to possess cytotoxicity against the cell lines. Of the tested compounds, the compound PZ-5 was found to possess significant cytotoxicity against the HT-29 cell line with an IC_{50} value of 27.64 µM.

Based on the IC_{50} value and the docking scores, compound PZ-5 was selected for further animal studies against DMH induced colon carcinoma on male Sprague Dawley rats.

Acute toxicity study was done according to OECD 423 guideline. No mortality was observed to a dose as 2000mg/kg p.o of (PZ-5) and the results showed that the compound could be used safely in the animals up to the dose of 2000mg/kg. Dose levels for the present study were chosen as 100mg/kg and 200mg/kg.

Experimental colon cancer induced by DMH in rats mimics human colon cancer and is therefore, used to evaluate anticancer property of drugs. In which, DMH will be converted to diazonium ions,azoxymethane(AOM) and methylazoxymethanol(MAM) in presence of NAD^+ -dependent dehydrogenase enzyme.These intermediates alkylate colonic mucosal DNA and results in oxidative stress followed by delayed repair of damaged DNA leads to the accumulation of multiple mutations such as Apc,K-ras,b-catenin and thus leads to susceptibility of specific adenocarcinoma of colon.

In this study, administration of DMH to the female Sprague Dawley rats produced significant colon tumour compared to normal control.

At the time of tumour development, animal body weight will be reduced. The intraperitoneal administration of DMH to the rats showed significant decrease in the body weight when compared to normal control rats. In the case of treatment groups after the administration of PZ-5 at different doses showed considerable prevention of weight loss when compared to DMH control rats.

Haematological Parameters

In DMH control rats, a significant decrease in the RBC and Hb levels were shown as compared to normal control rats. But the WBC level was increased. In the PZ-5 treated groups, the levels of RBC and Hb were increased and there is a significant reduction in the WBC level.

Biochemical Parameters

The biochemical parameters like SGPT, SGOT, urea, serum creatinine were estimated to detect the effect of PZ-5 on liver and kidney function. In the present study, a significant

increased serum ALP levels was observed in the DMH control rats when compared to normal control rats. Rats treated with PZ-5 showed significantly lower ALP levels when compared to DMH control. The other parameters were found to be non-significant when compared to DMH control rats.

***In vivo* antioxidants**

Living organisms have developed several effective mechanisms to get protection from the reactive oxygen species. The antioxidant defense mechanisms of the body include enzymes such as SOD,GSH, catalase and GPx.

Super Oxide Dismutase

The cells containing this enzyme scavenges superoxide ion and prevents its accumulation so that cells are protected from oxidative stress. SOD catalyses this dismutation of superoxide in which one O_2* is oxidized to O_2 while the other is reduced to H_2O_2. The formed H_2O_2 is able to damage the cellular components and are instantly removed by enzymes like catalase and GPx. A significant decrease in SOD levels was observed in DMH control rats when compared to normal control rats. The levels get increased in the rats treated with compound PZ-5 and 5-Fluoro uracil when compared to SOD levels in DMH control rats.

Catalase

Oxidative stress has a major role in carcinogenesis. The catalase enzyme is an endogenous antioxidant enzyme that neutralizes reactive oxygen species by converting H_2O_2 into H_2O and O_2. Among the other antioxidant enzymes, including superoxide dismutase and glutathione peroxidase, catalase is an elementary defense against oxidative stress. In DMH control rats, a significant decreased level of catalase was observed as compared to normal control rats. The compound PZ-5 and 5-Fluorouracil treated groups showed significant increase in catalase levels when compared to DMH control rats.

Reduced Glutathione

It prevents free radical induced oxidation of SH groups of various proteins to disulfide derivatives. It also protects haemoglobin from getting oxidized by H_2O_2. A significant decrease in the GSH levels was observed in the DMH control rats when compared to normal control rats. Rats treated with PZ-5 and 5-Fluorouracil showed significantly higher GSH levels when compared to DMH control rats.

Glutathione Peroxidase.

The biochemical function of GPx involves the reduction of lipid hydroperoxides to their alcohols and to reduce free hydrogen peroxide to water. Thus, it protects the cell from oxidative damage. The DMH control rats showed significant lower GPx levels when compared to the normal control rats. The compound PZ-5 and 5-Fluorouracil treated rats showed significant higher GPx levels when compared to DMH control rats.

Lipid Peroxidation

Lipid peroxidation indicates the oxidative degradation of lipids in which the free radicals steals electrons from the lipids in the cell membrane and leads to cell damage. This process proceeds by a free radical chain reaction mechanism. Radical reaction consists of initiation, propagation and termination processes. A significant increased level of LPx was observed in the DMH control rats when compared to normal control rats. The compound PZ-5 and 5-FU treated rats showed significant decreased LPx levels when compared to DMH control rats.

Mitochondrial TCA-cycle enzymes

As mitochondria plays a critical role in bioenergetics, anabolic and cell death pathways which range from high tissue-specific conditions to generalized whole-body disorders including cancer. Several common features of established tumour cells can directly or indirectly result from mitochondrial deregulation. Mitochondria may be implicated in early tumerogenesis.

Succinate dehydrogenase

The Succinate Dehydrogenase level was found to be significantly decreased in DMH control rats when compared to normal control rats. Rats treated with compound PZ-5 and 5-FU showed significantly higher Succinate Dehydrogenase levels when compared to DMH control rats.

Histopathological analysis

Histopathological analysis of tumours of various groups revealed that DMH control group shows dysplastic cells with occasional mitosis and high amount of infiltration whereas the level of infiltration for standard was less when compared with that of test compound PZ-5.

Immunohistochemistry analysis

COX-2 plays a pivotal role throughout oncogenesis mainly in colon carcinoma and is induced in response to growth factors. Immunohistolochemical analysis of colon tumours using COX2 as marker was performed. DMH control rats shows Cytoplasmic postivity in inflammatory cells (3+) whereas the standard and PZ-5 (high dose) shows Cytoplasmic postivity in inflammatory cells(1+).

8. CONCLUSION

In conclusion, some novel substituted Pyrazolo benzpyridazine derivatives have been designed and docked into target protein Human VEGFR2 (PDB ID 3VO3). Based on the docking score, feasibility of synthesis and chemical availability, six top ranked compounds with good dock score and binding interactions with amino acid cys919 were synthesized and characterized for their physical constants such as molecular formula, molecular weight, melting point, R_f value, and their chemical structures were confirmed by FT-IR, 1H-NMR, Mass spectroscopy. The synthesized compounds were evaluated for its *in vitro* and *in vivo* anticancer activity. All derivatives demonstrated mild to moderate *in-vivo* cytotoxic activity against HT-29 cell lines by MTT assay, out of which compound PZ-5 exhibited promising activity against HT-29 cell lines. In *in-vivo* anticancer screening studies of Compound PZ-5,it exhibited moderate activity against DMH induced colon carcinoma in Sprague Dawley Rats when compared with that of the standard 5-Fluorouracil.

Taking into account the significant activities of the examined compounds, it is believed that further optimization of these identified chemical leads can probably lead to the development of more active molecules. Further studies on its possible mechanism against HT-29 cell lines as well as against various other cell lines may also reveal significant activity against a wide variety of cancers.

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