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PYRIMIDINE DERIVATIVES TARGET CANCER THROUGH INHIBITION OF BRD4 EXPRESSION - RESEARCH ARTICLE

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ABSTRACT

Series of novel pyrimidine derivatives compound were designed with the help of chemsketch for its anti-cancer activity against bromodomain containing protein **(BRD4)** and protein was downloaded from protein data bank with PDB code **4HY3** using Accelrys drug discovery studio 3.5. among them The compound **2C** possess good CDOCKER Interaction energy, the compound **2c** was further synthesized and confirmed by the both physical data as well as spectral data analysis such TLC, Melting Point and IR, ^{1}H NMR, Mass spectroscopy,. The Cytotoxic activity of compound **2C** was assessed by MTT assay on various cancer cell line such as HL-60, Hela, MCF-7, A431 and HCT-116A. Acute toxicity was carried out based on OECD guideline 425, for *Invivo* model, therapeutic efficacy of compound 2c was evaluated against induced Ehrlich ascites carcinoma (EAC) cancer cell in Swiss albino mice. The animals were divided into 3 group, each group contain eight mice, Group I served as cancer control (0.9 % saline), Group II treated with 5 fluorouracil (20 mg/kg/p.o) can serve as a standard group, Group III treated with synthesized pyrimidine compound (200 mg/kg/p.o), All groups animals were induced with Ehrlich Ascites Carcinoma by injecting 1×10^{6} cells/ml/mouse (i.p) as well as therapy was provided orally once day for 10^{th} days and after post therapy, liver as well as kidney was isolated and antioxidant parameter was estimated, blood was withdrawn from the mice via retro orbital for haematological investigations. The

set of three groups containing two mice in each group was observed for the body weight analysis and life expectancy.

KEYWORDS: Antioxidant, Bromodomain, Catalase, Ehrlich Ascites Carcinoma, Pyrimidine.

1. INTRODUCTION

1.1 Introduction about cancer

Cancer is a disease that can damage cellular mechanism of almost all parts of the body when they get exposed to it, cancer cells may or may not invade to its neighboring normal cells. Due to lack of knowledge in early detection and insufficient target oriented treatment, till now it is very difficult to cure the cancer. There are several treatment available for the cancer like Chemotherapy, Gene Therapy, Laser Therapy, Angiogenesis Blockers, Biotherapies, Bone Marrow Transplants and Stem Cell Therapy. Among the others types of treatment, triple therapy including tumor surgery and platinum based chemotherapy are considered to be the most efficient treatment with the dose of bevacizumab, also the chalcones because of its maximum target site are available with several pathways.[1]

1.2 Pyrimidines

A six membered pyrimidine heterocyclic compound consist of two N-atoms in the ring, constitutes a significant component of nucleic acid, important pharmacophore for the synthesis of many novel drugs i.e., anticancer, antiviral and antibacterial agents.[2]

Pyrimidine based anticancer drugs have been developed based on structural modification of these core structures (i.e., substitution with moieties and rings, conjugation with other compounds, and coordination with metal ions). The pyrimidine derivative molecules shows potential as privileged scaffolds with attractive properties and biological activities for the search of novel anticancer agents.[3]

1.3 Bromodomains: Structure and Function

BRDs considered as the first identified protein that is coded with the D. melanogaster brahama gene, consist of 110 amino acid act as modulator throughout the evolution using various transcriptional co-regulators, chromatin modifying enzymes including nuclear scaffold proteins and directly bind to histone residue of acetylated lysine via NF-kappa B subunit RelA.[4]

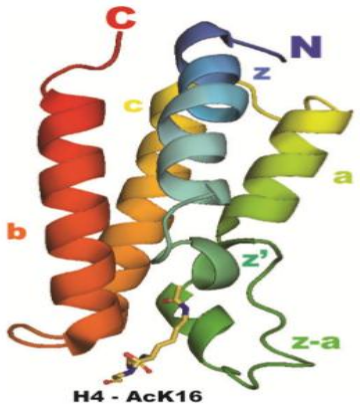


Fig.1: BRD4 bromodomain 1 structure showing the binding with the acetylated Lysine16 on the tail of Histone H4.

The BRD structure consists of a left-handed bundle of four antiparallel alpha helices linked by two loop regions (Fig. 1) the co-crystal structures of BRDs bound to acetyl-lysine containing peptides, the acetylated lysine is first recognized in a hydrophobic pocket located between the two loops that form most highly conserved residues. The asparagine at the core of the binding site, engages the acetyl-lysine via a hydrogen bond between its NH2 group and the acetyl carbonyl oxygen atom of the acetylated lysine. At the entrance of the binding pocket, the two loop regions interact with adjacent residues to the acetylated lysine in the target sequence. Although the acetyl-lysine binding pocket for all BRDs is hydrophobic, there is considerable variation in the electrostatic interactions at the opening of the pocket among BRD families.[5]

This variation determines the specificity of individual BRDs and provides the opportunity to both sub-classify BRD proteins into families and develop specific small molecules that are specifically targeted against certain families. Bromodomain-containing protein 4 (BRD4), firstly identified in 1988 as a subset of epigenetic “reader”, is a member of the bromodomain and extra-terminal (BET) protein family.[6] In the past few years, compared with other BET family proteins (BRD2, BRD3 and BRDT). The BRD4 has been most extensively studied because of its unique relevance with a number of human diseases including cancer, inflammation, cardiovascular diseases and multiple organ fibrosis.[7-9] Normally, BRD4 localizes in the nucleus and recruits positive transcriptional elongation factor complex (P-TEFb) to regulate RNA polymerase II activity, via binding to ε-N-acetylated lysine residues of histon.[10-12]

In this way, BRD4 directly control downstream gene expression, which are responsible for cellular proliferation and inflammatory pathways. For instance, BRD4 promote transcription of the cMyc oncogene and further increase the expression of c-Myc targeting genes lead to cancer pathogenesis.[13-17] In addition, BRD4 reported to play important roles in fibrosis via directly binding to the enhancer of collagen-I, the main inducement of fibrosis**.** Therefore, targeting BRD4 would be a potential strategy in cancer and fibrosis therapy.**[10-18]**

1.3.1 Mechanisms and consequences of Brd4 dysregulation in cancer

The actual mechanism of bromodomain in cancer progression are unidentified till now. Though the bromodomain protein abundantly present in the cell, help in the normal growth, regulation and differentiation of the cell in the body. the deregulation of protein contribute to cancer via through chromatin remodeling and gene transcription.**[19]** BRD4 lead to human cancer that are reported due to gene mutations, the missense and nonsense substitution.**[20]** Aberrant expression of BET proteins, promotes the cell cycling progression, invasion and metastasis of cancer cell lines *in vitro*. The BRD4 are present in the squamous cell carcinoma in the fusion form which enhanced cell invasion and migration *in vitro*.**[21]**

1.3.2 Epigenetic regulation of gene expression by Brd4 in the tumour microenvironment

Tumors consist of a heterogeneous population cell that include both the neoplastic tumor cells and non-neoplastic cells that form the tumor microenvironment. The tumor microenvironment has various types of cells including immune cells, fibroblasts, inflammatory cells that are originated from the bone marrow and endothelial cells which promote the growth of the tumor blood vessels. Continuous signaling and molecular cross-discussion occurs within tumor microenvironment the between tumor cells and normal collectively, the molecular signalling events occurring within tumor microenvironment, promote tumor progression and allow cancer cells to acquire phenotypic.**[22-41]**

1.3.3 Therapeutic strategies to target bet bromodomain proteins in cancer

It was found that normal regulation of the BET family containing protein help in the proper functioning of the biological system but dysregulation contributes to the initiation, progression and metastatic activity of cancer cells.**[42]** Most notably, maintenance of the malignant phenotype in cancer cells is partly dependent on epigenetic deregulation in both hematopoietic and solid tumor cells.**[43]** This reliance on epigenetic proteins makes a therapeutic target promising. As such, therapies aimed at targeting epigenetic regulators and inhibiting the role of BET protein can represent a novel cancer therapy strategy. The small

molecule inhibitors, inhibits the BET proteins *in vitro* anti-tumor properties.[44] both *in- vitro* and *in-vivo* show potential therapeutic effects in phase I clinical trials performed in human cancer patients.[45]

2.0 MATERIALS AND METHOD

2.1 Docking studies

Docking program requires three computation steps to carry out docking study these are as follows:

(1) Preparation of the receptor

(2) Preparation of the ligand

(3) Setup of the parameters of the docking program

The following subsections describe these three steps in detail.

2.2 Receptor preparation

The three dimensional structure of BRD4 (PDB CODE-4HY3) were obtained from PDB. (http://www.rcsb.org/pdb/home/home.do). RCSB is a single, global archive for information about the 3D structure of macromolecules such as protein, DNA and their complexes, as determined by X-ray crystallography, NMR spectroscopy and cryoelectron microscopy.[46]

2.3 Ligand preparation

The pyrimidine derivative compound were designed with help of chemdraw and the ligand were loaded into Accelrys drug discovery studio 3.5. To predict the ligand molecular properties e.g. a log P value, hydrogen bond donors and hydrogen acceptors, surface area and molecular weight, absorption, distribution, metabolism (ADME) and analyses for solubility, intestinal absorption excretion and toxicity. High throughput screening approaches and virtual screening were used for the identification of lead compounds. The compound datasets were screened effectively in the initial stages for ADMET to decrease cost and clinical failures of new drugs.[46]

2.4 Molecular simulation studies

Chemistry at Harvard Molecular Mechanics (CHARMM) force field is a flexible molecular mechanics and dynamics program that are used in drug Accelrys drug discovery studio 3.5. For ligand minimization and protein minimization, broad range calculations

such as calculation of geometries, interaction and conformation energies, local minima, barriers to rotation, free energy time-dependent dynamic behavior, and simulations.[46]

2.5 Target protein and active site prediction

The various literature surveys were taken into consideration for the evaluation of protein and the active sites.

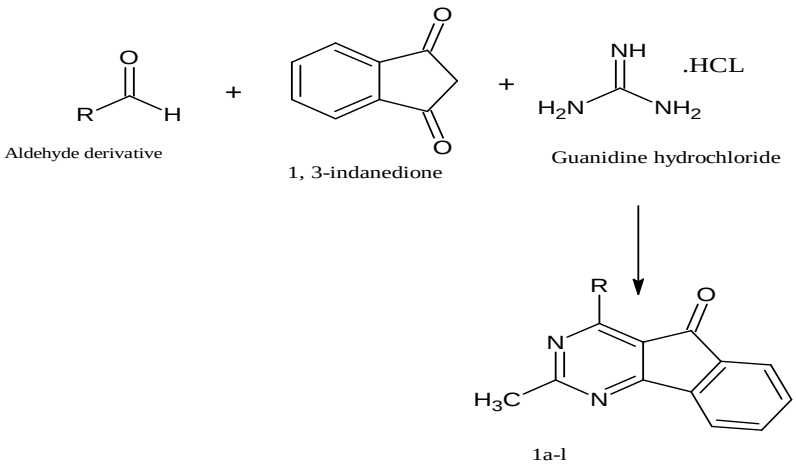
2.6 Molecular docking

To carry out docking study, Accelrys drug discovery studio 3.5 are used. In this study, ligand were designed using chemsketch/chemdraw and protein were downloaded from protein data bank (PDB) with the link (http://www.rcsb.org/pdb/home/home.do). E.g. to download bromodomain protein 4HY3 is the PDB code. Hydrogen were added to interact with amino acid present in the particular protein which is seen in 2D structure. To add the hydrogen click on chemistry then hydrogen add. Both the ligand and protein should prepared. Ligand were prepared on clicking small molecule followed by prepare ligand and then ligand minimization were done. Protein preparation were done on clicking macromolecules then prepare protein followed by full minimization of protein once both the ligand and protein were prepared the click on receptor ligand interaction, List will be display, click on define and editing binding sites, click on receptor cavities click on docking ligand (C-Docker), Box will appear (In parameter value), Input receptor = 4hy3, Input ligand = add all the ligand. Click on run.[46]

2.7 Schematic representation: Step involve in synthesis of pyrimidine derivatives compound

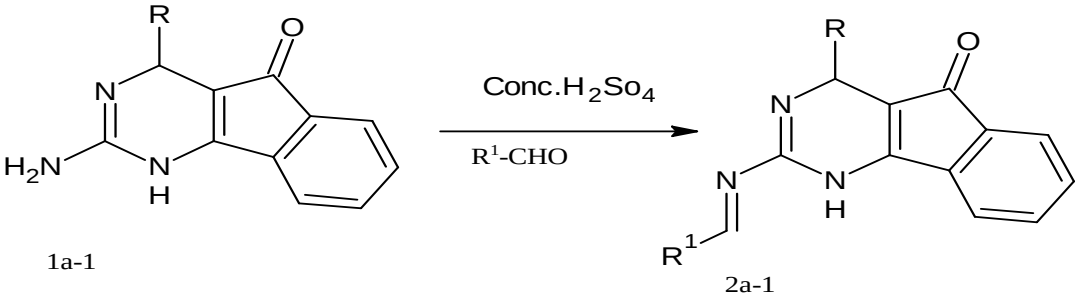
GENERAL PROCEDURE

Step 1: synthesis of 2-amino-5H-indeno [1, 2-d] pyrimidine -5-one derivative.



The aromatic substitution aldehyde containing (8 mmol) and 1, 3-indanedione containing (8 mmol) using solvent such as water- ethanol in the ratio of 15:20 ml with 2-5 drops of conc. NAOH in the round bottom flask were added and the resultant mixture were stirred properly at the room temperature. Guanidine HCL containing (16 mmol) was added in the same round bottom flask and refluxed for 2 h monitored by TLC. The obtained reaction mixture were poured slowly in crushed ice with constant stirring, formed product 1a-l was isolated with the filtration and the excess amount of sodium hydroxide were remove from 1a-l by washing with the ice cold water.[47]

Step 2: Synthesis of 2-(methylideneamino) - 5H-indeno [1, 2-d] pyrimidine-5 one derivatives-2(a-1).



1a-1 2a-1

Once the product **1a-1** were formed, the compound **1a-1**(6 mmol) were substituted with aromatic aldehyde (6 mmol) in the iodine flask and solvent water- ethanol in the ratio of 15:20 ml and few drop of conc. sulphuric acid were added in the same iodine flask and were refluxed for 2 h and monitored by TLC once the reaction get completed. Reaction mixture were poured slowly in crushed ice with constant stirring, filtered and solid were taken and filtrate were discarded, dried followed by recrystallization with ethanol and the obtained final product **2a-1**.[48]

2.8 INDENTIFICATION AND CHARACTERIZATION

The compound synthesized were identified and characterized by following methods such as

- Melting point determination
- Thin layer chromatography
- Infra-red spectroscopy
- Nuclear magnetic resonance spectroscopy
- Mass spectroscopy

2.8.1 Melting point determination (MP)

The melting point of organic compound was determined by open capillary tube method. The determination of melting point is the most important and easiest way of differentiating this physical constant of one compound from other.[49]

2.8.2 Thin layer chromatography (TLC)

The precoated TLC plate made of silica gel F_{254} was used to determine the R_F value. TLC is an important method for synthetic chemistry to infer the formation of compound based on the R_F value. It also help in confirming the progress of the reaction. The solvent used was Pet ether: ethyl acetate (1:1).[49]

2.8.3 Infra-red spectroscopy (IR)

The infrared spectra were recorded by FT-IR technique in tensor 27 spectrophotometer, Brucker optic (Germany) using ATR technique. IR is one of the most important tools for determining the various functional group and the possible chemical structure. The important advantage of IR over the other technique is that it provide finger prints (1300-650) cm^{-1} giving information about the structure (functional group, bonding with Each other) of molecules easily. This technique is based upon the molecular vibration of the compound such that each and every bond will vibrate at different frequency and when this vibration frequency correspond to IR frequency, Spectra of each and every bond will be formed.[50-51]

2.8.4 Nuclear magnetic resonance spectroscopy (NMR)

The NMR Spectra of selected compound were recorded in DMSO (internal standard TMS-tetra methyl silane) at 400 MHz on the AMX 400 at the sophisticated instrument facility (SIF), Dept. of physic. IISC, Bengaluru and Interpreted for the aromatic /heterocyclic proton (delta values).Proton NMR is one of the most important tools for determining the various proton present in structure. The interaction between compound and electromagnetic force can be observed by subjecting a substance simultaneously to two magnetic forces, one other varying at some radio frequency. At a particular combination of field, energy is absorbed by the sample and absorption can be observed as a change in signal developed by radio frequency detector and amplifier. This energy of absorption can be related to a magnetic dipolar nature of the spinning nuclei. This technique is known as Nuclear Magnetic Resonance. This technique is useful in determining the structure of the molecules.[50-52]

2.8.5 Mass spectroscopy

Mass spectra were recorded using a joel-D-300, Mass spectrophotometer (70ev), shimadzu (japan) by Q- TOF. Mass spectroscopy is a technique through which molecular characterization is carried on bombarded with high energy beam of electrons. Mass spectra is very much essential to elucidate or interpretate molecular weight and molecular structure of a compound.

The physic behind the mass spectrometry is that the charge particle gets deflected on passing through the magnetic fields in the circular path of radius and directly proportion to mass to charge ratio (m/e). The molecular ion are formed upon displacement of electron from its organic molecules with a high beam of energy. When the molecular ion are less stable then fragment of ion are very small. The magnetic field accelerate the ion and help to deflected in the circular path, according to the masses of the ions.by adjusting the magnetic field, the ion can be focused on the detector and recorded.[50-52]

2.9 *IN VITRO* STUDIES

2.9.1 MTT assay

Methods

A cell culture are carried out with different cancer cell line such as HELA, HL-60, MCF-7 The cancer cell line are maintained in the logarithmic phase of growth with RPMI-1640 medium, supplemented with heat-inactivated 10% fetal bovine serum (FBS) and 1 % penicillin/streptomycin incubated in 5 % CO_2 incubator and 95 % humidified air. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] is the quantitative standard colorimetric assay used to measure the mammalian cellular growth, cell survival and cell proliferation based on ability of cell a live. This assay determine the cytotoxicity of potential medicinal agents and other toxic materials. The pale yellow of MTT enter inside the mitochondria of viable cell and get reduce enzymatically to form dark color formazan crystal by cleaving tetrazolium rings. Though the formazan crystal are insoluble in aqueous solution. Therefore it is treated with organic solvent like acid-isopropanol (0.04 N Hydrochloric in isopropanol) to dissolve and produce purple formazan product (color solution) and the absorbance were taken from 490 nm to 600 nm by using the ELISA reader.[53-55]

Procedure

- 10 ml of the culture cell suspension was taken in 15 ml tubes and were centrifuged at 2500 rpm for 10 min.

- The supernatant was discarded and the cell pellet was resuspended in growth medium (10% fetal bovine serum (FBS) and 1 % penicillin/streptomycin incubated in 5 % CO_2 incubator) for 24 h
- The cell viability was checked by counting the numbers of viable cells in the above 1 ml suspension through hemocytometer and diluted the resuspended cells with growth medium to get required cell concentration.
- 1×10^4 exponentially growing cells were seeded per well in 96 well plates.
- Cells were exposed to various concentrations of drug.
- The plates were incubated at 37 °C in 5 % CO_2/95 % humidified air.
- 100 µl of growth medium and 10 µl of MTT was added (5 mg/ml) into each well of 96 well plates and plates were incubated at 37 °C in 5 % CO_2/95 % humidified air for 4 h.
- The precipitated formazan salt was dissolved to form a colored solution by adding 100 µl of acid-isopropanol (0.04 N HCl in isopropanol) into each 96 well plate.

  The absorbance of this colored solution was measured at a wavelength of 570 nm using a multiwell scanning spectrophotometer (ELISA reader).[53-55]

Percentage cell viability was calculated using the equation

$$\% \text{ Cell viability} = \frac{\text{Absorbance of cells (treated)}}{\text{Absorbance of cells (untreated)}} \quad X\ 100$$

2.9.2 *In-vitro* BRD4 enzyme inhibition assay

Method

The kits assay human BRD4 level in the samples, use purified human BRD4 antibody to coat microliter plate well, make solid- phase antibody then add sample (containing human BRD4) to well, combined human BRD4 antibody which with HRP leveled become antibody-antigen-Enzyme- antibody complex, after washing completely, Add TMP substrate solution, TMP substrate become blue color at HRP enzyme, catalyzed, reaction is terminated by the addition of sulphuric acid solution and the color change (yellow) is measured Spectrophotometrically at the wavelength 450 nm. The concentration of human BRD4 in the sample is then determined by comparing the O.D. samples to the standard curved. The best compound selected for *in vitro* brd4 inhibition assay. Enzyme inhibition studies was carried out as per the instruction of the ELISA kit (catalogue number E13652167).[56]

Percentage inhibition of BRD4 was calculated.

The equation of percentage inhibition.[56]

% inhibition= [{control (abs) –Test (abs)} ÷ control (abs)}] ×100

Cell lines

Ehrlich Ascites Carcinoma (EAC) cancer cells were obtained from Amala Cancer Research Centre, Thrissur, Kerala 680 555.

2.9.3 *IN VIVO* STUDIES

Drug solution preparation & Dose selection

Drug solution preparation

The synthesized pyrimidine derivative molecules were tested at doses of (200 mg/kg) body weight of mice. The molecules were dissolved in water with a pinch of acacia appropriately and the drug will be prepare freshly and 1ml of drug suspension was given to each mice for test animals.

Dose selection

Acute toxicity study for synthesized pyrimidine derivative compound were done by adapting up & down method, Committee for the Purpose of Control and Supervision on Experiment on Animals (CPCSEA) organization of economic cooperation and development (OECD) guidelines 425. The test was carried out by using AOT425Statpgm (Version: 1.0), and the test results and recommendation were based on the acute oral toxicity (OECD of guidelines 425) statistical program**.** The Swiss albino female mice whose body weight 20-25 g were taken to carried out acute toxicity study. The animal were given all the required facility as per protocol of the experiment. The pyrimidine derivative compound were given orally and animals were observed continuously for 12 h to detect any change in autonomic or behavior response and continue for 24 h. mortality rate was observed for 48 h. as per the main test, the study was started with 175 mg/kg, followed by 550 mg/kg and completed with Administration of 2000 mg/kg p.o with a limit dose of 2000 mg/kg. Observed the animal for 14 day for any toxicity sign.[57]

Experimental method

Healthy Swiss albino female mice whose body weight 25 ± 3 g were procured from Central Animal Facilities, Acharya & BM Reddy College of Pharmacy, Bengaluru. Animals were housed in polypropylene cages and maintained under standard conditions. They were fed

with standard diet and water as per protocol. The animals were maintain as per CPCSEA (Committee for the Purpose of Control and Supervision of Experimental Animals) guideline for the care and use of laboratory animals. The study protocol number IAEC/ABMRCP/2018-2019/20 was approved by Institutional Animal Ethics Committee (IAEC), Acharya & BM Reddy College of Pharmacy, Karnataka Bengaluru.

Cancer cell count and induction

1 ml of normal saline (0.9 %) was injected intraperitoneally into donor mouse. After injecting saline, immediately 1 ml of ascites fluid was collected from peritoneal cavity and diluted with normal saline upto 10 ml. 10 µl ascites fluid from this was taken and placed on Neubauer's chamber and the number of cells appeared on chamber were calculated and concentration of 1×10^6 cells were injected to each mouse intraperitoneally.

Calculation

Area of one square of WBC chamber (A) = 0.04 mm

Depth of one square of WBC chamber (D) = 0.1 mm

Volume of one square of WBC chamber (V) = Area x Depth = 0.04 mm^3 x 0.1 mm = 0.004 mm^3

Total volume of 4 squares of WBC chamber = 4 x 0.004 mm^3= 0.016 mm^3

Total cancer cells to be induced = (Total number of cancer cells in all 4 squares of WBC chambers (X) divided by total volume of all 4 squares of WBC chambers) multiplied by dilution factor.

Dilution factor = 10

Total number of cancer cells counted in all 4 square = 400 cells.

Total volume of 4 chamber = 0.4 mm^3 or 0.4 µl or 0.0004 ml. That is, 0.0004 ml contains 400 cancer cells.

Total cancer cells present per ml = 400 × 10 = 0.0004 = 1×10^7 cells/ml

Total cancer cells present in 0.1 ml = $1\times 10^7 \times 0.1 = 1\times 10^6$ cells

Therefore, 0.1 ml of above concentration was injected to each mouse intraperitoneally.

Normal saline upto 10 ml. 10 µl ascites fluid from this was taken and placed on Neubauer's chamber and the number of cells appeared on chamber were calculated and concentration of 1×10^6 cells were injected to each mouse intraperitoneally.

2.9.3.1 TREATMENT PROTOCOL

Ehrlich ascites carcinoma model

0.2 ml of 24 Adult healthy Swiss Albino female mice are inoculated with Ehrlich Ascites Carcinoma (EAC) cells and divided into three different groups, each containing 8 mice in a group. After tumor inoculation, treatments will be given orally, once daily for 10 days. On the 11th day, six animals from each group will be anaesthetized and blood will be collected through tail vein method. The hematological parameters like white blood cells (WBC), red blood cells (RBC), hemoglobin (Hgb) and platelets (PLT) will be estimated. The ascetic fluid will be collected and measured the tumor cell packed volume and viable tumor cell counts. The rest of the animals will be kept to check average life span (ALS), percentage increase in life span (%ILS) and body weight analysis.[58-60]

Table 2.9.3.2 Treatment Protocol

The study protocol number **IAEC/ABMRCP/2018-2019/20** was approved by Institutional Animal Ethics Committee (IAEC), Acharya & BM Reddy College of Pharmacy, Bangalore, and Karnataka 560-107

Groups	Treatment	Dose	No. of mice
1.	Cancer control	0.9 % normal saline	8
2.	5-FU(standard)	20 mg/kg	8
3.	Pyrimidine derivative (test)	200 mg/kg	8

2.9.3.3 Estimation Using Blood Sample

Determination of hematological parameters

In order to know the effect of the Synthesized Pyrimidine derivatives on hematological status of EAC cells bearing mice, a comparison between Group I (Cancer control), Group II (Standard) & Group III (Synthesized Pyrimidine derivatives/Test) was done. Blood was drawn from each mice by retro-orbital plexus method and was collected in 12 µl of EDTA tube, for the Hematological studies and this blood samples were subjected to Animal Blood Counter (blood all count) for RBC count, WBC count and the hemoglobin content.[61-62]

Measurement of mean survival time (MST) and Percentage increase in life span (% ILS)

The effect of pyrimidine derivative compound (200 mg/kg) on tumor growth was completely monitored and then recorded for their mortality rate daily until all the animals were dead and % ILS was calculated by using the formula.

% ILS = [MST of treated group/MST of control group -1] × 100 %

Body weight analysis: All the mice were weighed weekly after tumor cell inoculation and the average increase the body weight of the carcinoma induced mice was measured and the percentage decrease in the body weight was determined by using formula.[62]

Percentage decrease in the body weight

$$\frac{(Gc - Gt) \times 100\ \%}{Gc}$$

Where,

Gc = gain in the body weight of control group.

Gt = gain in the body weight of treatment group.

2.9.3.4 Estimation Using Tissue Homogenate

Method

After sacrificing of the animal, liver tissue was removed and washed with cold saline, kept on ice and subsequently blotted on filter paper. Then weighed and 10 % of the tissue homogenate were prepared by taking 1 g of liver tissue with 10 ml PBS ice-cooled (0.2 M, pH 7), homogenized at 4000 rpm. The homogenization procedure was performed as quickly as possible. The homogenate was centrifuged at 10.000 rpm at -4 °C for 15 min. The supernatants were collected to carry out different biochemical estimations (SOD, GSH, catalase, nitric oxide and total protein) at ABMRCP, using Agilent Cary 60 UV-Vis spectrophotometer, supported by GLP software, optional 21 CFR parts 11 capable software, and dedicated instrument validation software which included pharmacopeia test suites.

2.9.3.5 Superoxide dismutase (SOD) estimation

Procedure

In 1984, Kakkar et al developed the Superoxide dismutase activity method. In this method 1.2 ml of 0.052 M sodium pyrophosphate buffer (pH 8.3) was prepared and the obtained 0.1 ml of the supernatant which was centrifuges before were added along with 0.1 ml of 186 µM phenazonium methosulphate, 0.3 ml of 300 µM Nitroblue tetrazolium and 0.2 ml of 780 µM NADH solution. The above reaction mixture was incubated for 90 sec at 30 °C to stop the reaction 0.1 ml glacial acetic acid were added and then 4 ml n-butanol were also added, the reaction mixture were centrifuged at 4000 rmp and 4°C for 10 minutes, at 560 nm against the blank the absorbance of organic layer was measured. One unit of the enzyme activity is defined as the required concentration of enzyme to inhibit the absorbance of chromogen production by 50 % in control sample under the assay conditions.[63]

$$\text{Formula: SOD (IU/mg)} = \frac{\text{(Blank Abs. – Sample Abs.)}}{\left[\frac{\text{Blank Abs}}{2}\right] \times \text{Total protein}}$$

2.9.3.6 Reduced glutathione (GSH) estimation

Procedure

1.5 ml of 0.2 M Tris buffer (pH 8) and 0.1 ml of 0.01 M DTNB was added to 0.5 ml of the supernatant, the mixture was brought to 5 ml with 2 % SDS solution. Blank reagent without sample supernatant was prepared in a similar manner. The UV light absorbance of the clear supernatant of mixture was read at 412 nm.[64]

Formula

$$\text{GSH (mM/g)} = \frac{\text{Abs. at 412 nm} \times \text{D.F} \times 1000}{\epsilon}$$

Where ϵ = Extinction coefficient =13600 M^{-1} cm^{-1}

2.9.3.7 Catalase (CAT) estimation

Procedure

Catalase activity was measured by the method of Aebi et al., 1974.[65] Tissue homogenate supernatant (0.1 ml) was added into a test tube containing 1.9 ml phosphate buffer (50 mM, pH 7), and then 1.0 ml of freshly prepared H_2O_2 (30 mM) was added. Change in absorbance at 240 nm, was read at the 30th sec and the 3rd min after the addition of H_2O_2. The reaction mixture without adding a supernatant of tissue homogenate was used as blank. One unit of the enzyme activity is defined as enzyme concentration required, inhibiting the change in the absorbance by 50% in one min in the control sample.[65]

Formula

$$\text{CAT (IU/mg)} = \frac{\Delta \text{ Abs. (Change in absorbance)} \times \text{Volume of reaction mixture}}{43.6 \times \text{Volume of Enzyme added} \times \text{Total protein}}$$

2.9.3.8 Nitric oxide (NO) estimation

Procedure

In 1994, Marcocci et al. developed the method based on the nitric oxide scavenging activity.[66] in this method 1 ml of 25 mM sodium nitroprusside solution, 4 ml of test sample (supernatant) were added in the clean dry test tube and incubated at 37 °C for 3 h, from

incubated solution, 1.5 ml was taken in another clean dry test tube to that 0.9 ml of Griess reagent (1% sulphanilamide in 2 % 0.1% NEDA) were added with proper shaking, chromophore were formed due to the diazotization of the nitric acid with sulphanilamide further that get coupled With the Naphthylethylenediamine dihydrochloride and the absorbance was immediately measure at 540 nm. Control was prepared without the test sample.[66]

Formula

The percentage inhibition of radicals by the test sample was calculated using the formula:

NO inhibitory ratio $= A_{control} - A_{sample} \times 100 / A_{control}$

Where

$A_{control}$ = Absorbance of the control in the absence of sample

A_{sample} = Absorbance of sample

2.9.3.9 Estimation of total protein

Through osmotic pressure, tissue protein is involved in the maintenance of the normal distribution of water between blood and tissue. Several fractions of tissue protein vary independently and widely in a disease condions.[67]

Procedure

1000 µl of reagent was mixed to 20 µl of sample in Eppendorf tube. This mixture was aspirated and absorbance was measured directly at 540 nm, using Semi-Automatic analyser (BTS-350).

Statistical analysis

All the data are expressed as Mean ± SEM (n=8) Data were analyzed by GraphPad Prism 7 software and the parameters were analyzed by one way ANOVA followed by Dunnett's t-test for multiple comparisons, $P<0.001$ (***), was taken as significant.

3.0 RESULT AND DISCUSSION

Table 3.1 Result of the docking study of pyrimidine derivatives with bromodomain receptor.

Compound Code	(-) CDocker_ Interaction_Energy	Interaction Ligand_Residue	H-bond distance in Å	Interacting amino acids
JQ1	28.97	-	-	-
2a	25.26	-	-	-
2b	24.52	-	-	-
2c	29.80	Attached to OH	2.30479	ARG 16
2d	23.32	-	-	-
2f	26.42	Attached to OH Attached to OH	2.05715 2.57332	ARG 16 SER 83
2g	25.61	-	-	-
2h	25.69	Attached to OH	2.85763	ARG 16
2i	25.06	Attached to OH	1.92667	ARG 16
2j	27.73	Attached to OH	2.45174	ARG 16
2k	28.40	Attached to OH Attached to OH	1.89613 1.99504	ILE 21 ARG 16
2l	28.49	Attached to OH	2.53887	ARG 16
2m	28.92	Attached to NH	2.11062	ILE 21
2p	25.77	-	-	-
2q	26.22	-	-	-
2r	25.03	-	-	-
2s	29.02	Attached to OH	1.83902	ARG 16
2t	25.78	-	-	-
2u	24.61	-	-	-

fig. 2D structure of lead compound and standard (JQ1) compound

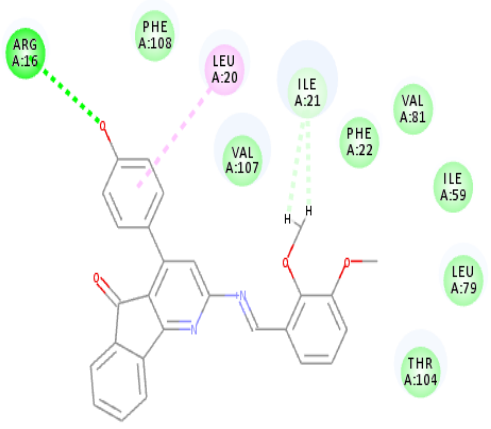


Fig. 2 Binding interactions between 2C with BRD4

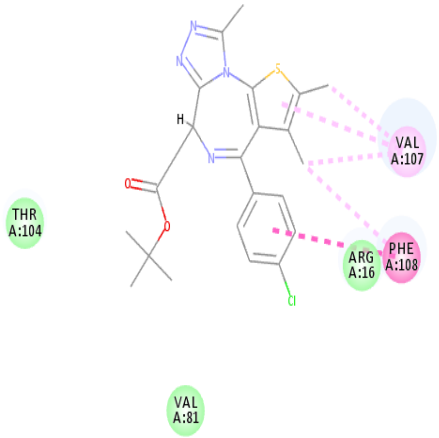


Fig. 3 Binding interactions between JQ1 with BRD4.

Molecular docking study was carried out for methoxy substituted pyrimidine derivatives compound and standard compound JQ1 inhibitor for bromodomain receptor using Accelrys discovery studio 3.5 with PDB Code **4HY3**. It was found that compound **2c, 2f, 2h, 2i, 2j, 2k, 2l, 2m and 2s** showed good CDOCKER interaction energy ranges from **-29.80 to -25.06 kcal/mole** and compound **2f, 2h, 2i, 2j, 2k, 2l, 2m** and **2s** interact with **ARG 16, SER 83 and ILE 21,** Among them compound **2c** possess good CDOCKER interaction energy with bromodomain receptor and interact with **ARG 16** with hydrogen bond distance **2.30 Å** and hence it is expected to bind with the receptor more effectively than other compounds. Whereas the standard compound (JQ1 inhibitor) doesn't possess any interaction with any of the amino acid. Thus, as compared to standard compound JQ1 inhibitor, Compound **2c** shows better anti-cancer activity. Therefore, docking study indicated that the compound **2c** binds with important amino acid residues present in the receptor. Hence we hypothesized that the designed pyrimidine derivatives can be an inhibitor of bromodomain.

Table 3.2: Docking score with different cancer cell line.

Cell Line	PDB Code	(-) CDocker_ Interaction_Energy	Interaction Ligand_Residue	H-bond distance in Å	Interacting amino acids
HELA (Cervical cancer)	3MXF	11.1537	Attached to OH Attached to OH Attached to OH	1.87721 1.96682 2.15191	TYR 97 MET 105 MET 132
HCT-116 (Colon Cancer)	4PY6	10.1716	Attached to OH Attached to CH	1.82449 2.92817	TYR 1235 ILE 1270
MG63(human osteosarcoma)	5Z5U	26.1494	Attached to NH	2.00162	GLN 85
Mcf-7 (breast cancer)	6DJC	51.2443	Attached to OH Attached to NH	1.93027 2.17997	MET 105 PRO 82
PA-1(ovarian teratocarcinoma)	6FSY	37.5743	Attached to NH	2.59637	PRO 82

Docking study was carried out with compound **2c** to inhibits different cancer cell line such as **HL-60** (Leukemia cancer cell line), **HELA** (Cervical cancer cell line), **MCF-7** (Breast cancer cell line), **HCT-116** (Colon cancer cell line), and **A 431** (Squamous carcinoma cell line) which occur due to over expression of bromodomain receptor using Accelrys discovery studio 3.5. The compound **2c** inhibits all other the cancer cell line but more efficiently inhibits **MCF -7** cancer cell line having CDOCKER interaction energy **51.2443** with hydrogen bond distance of **1.93027Å, 2.17997Å** and interact with amino acid **MET 105 and PRO 82.**

3.3 2D image of docked ligand with different cancer cell line

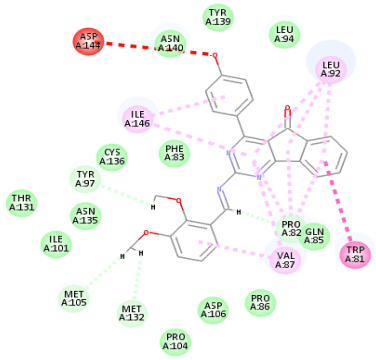


Fig. 4 Binding interactions between 2c with HELA

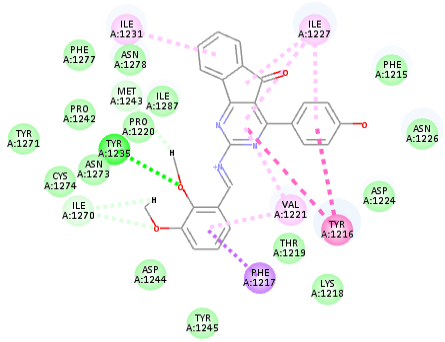


Fig. 5 Binding interactions between 2c with HCT-116

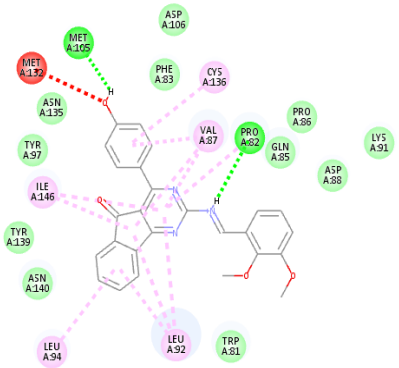


Fig. 6 Binding interactions between 2c with MG63

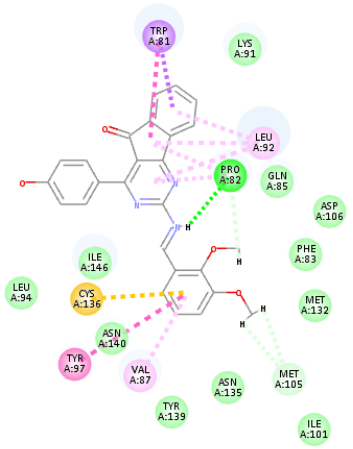


Fig. 7 Binding interactions between 2c with PA-1

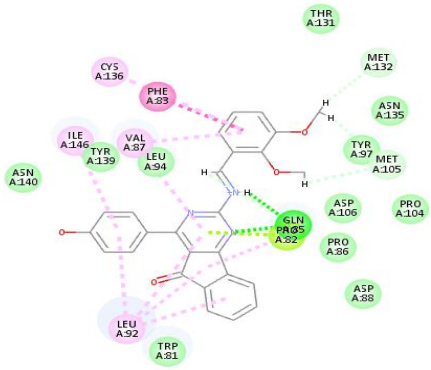


Fig. 8 Binding interactions between 2c with MCF-7.

3.4. ADME investigation

Accelrys drug discover studio 3.5. was used to calculate in-silico ADME parameters. They'd been calculated to avoid malfunction of the drug within the last phases of drug discovery process. All the designed 28 compounds possessed show good ADME properties. The aqueous solubility level 0 and 1. for 0 level value is (log(molarsolubility) <−8.0) that show

extremely low whereas 1 level value is −8.0 < log(molar solubility) <−6.0) which indicate no, very low but possible and BBB level 1 and 4 the value 1 indicate high and the value 4 show undefined. Inhibition level of CYP2D6 were1 and less 1. The 0 indicate Non-inhibitor and 1 show inhibitor. hepatotoxic level were less than 1 that show non toxic. All these recommended that the designed compounds could be druggable and hence it'd been more ready for docking studies. The details of the ADME exploration had been specified in **Table 3.4.**

Table 3.4 : ADME Study of the designed pyrimidine compound

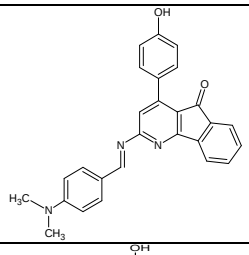
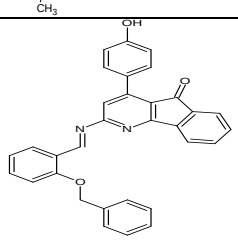
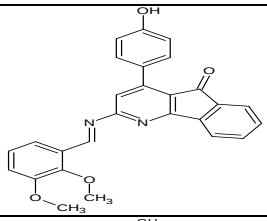
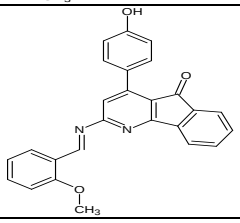
Compound code	Compound structure	Aqueous solubility Level	BBB Level	CYP2D6	Hepatotoxicity Level	PPB Level
2a		1	4	-1.50	-4.54	4.23
2b		1	4	-1.41	-1.67	2.11
2c		1	4	1.95	-2.75	3.56
2d		1	1	-0.70	-2.04	2.98

Table 3.4 : ADME Study of the designed pyrimidine compound

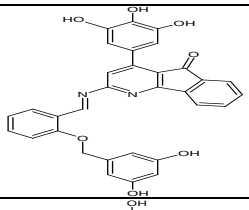
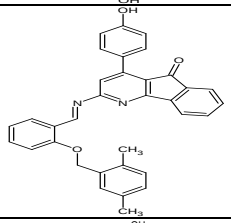
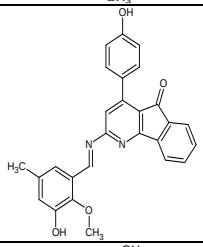
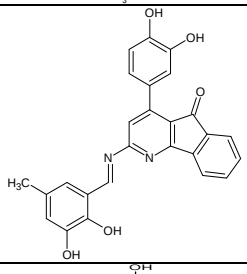
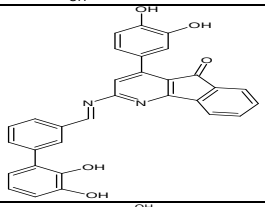
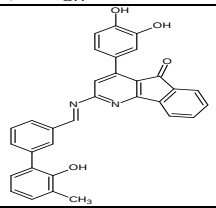
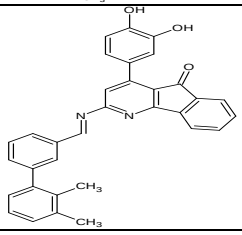
Compound code	Compound structure	Aqueous solubility Level	BBB Level	CYP2D6	Hepatotoxicity Level	PPB Level
2f		1	4	-1.07	-2.01	0.26
2g		0	4	0.28	-0.31	3.49
2h		1	4	-2.74	-2.02	3.06
2i		1	4	-2.62	-3.01	2.65
2j		1	4	-1.99	-4.33	1.99
2k		1	4	-2.01	-3.93	3.18
2l		1	4	-0.70	-1.98	4.54

Table 3.4 : ADME Study of the designed pyrimidine compound

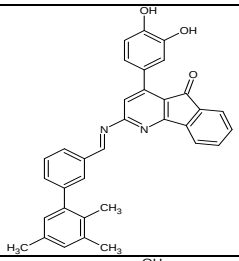
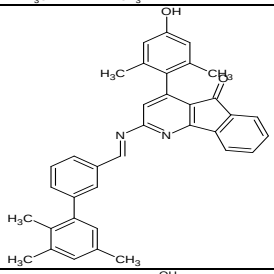
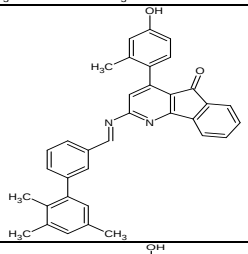
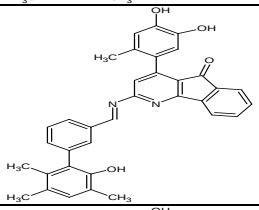
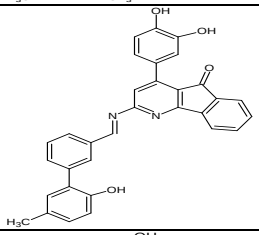
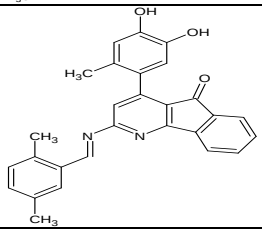
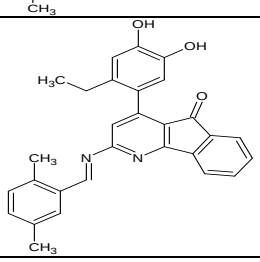
Compound code	Compound structure	Aqueous solubility Level	BBB Level	CYP2D6	Hepatotoxicity Level	PPB Level
2m		0	4	-1.50	-1.72	4.37
2p		1	4	-1.10	-1.60	5.70
2q		0	4	-1.17	-1.27	5.42
2r		0	4	-2.71	-3.28	2.83
2s		1	4	-2.01	-2.95	1.87
2t		1	4	-1.36	-2.21	3.29
2u		1	4	-1.61	-1.35	3.89

Table 3.4 : ADME Study of the designed pyrimidine compound

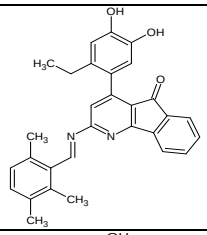
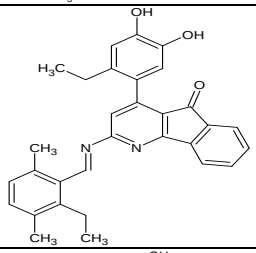
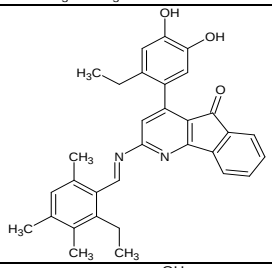
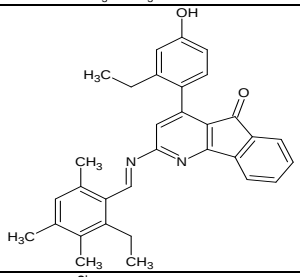
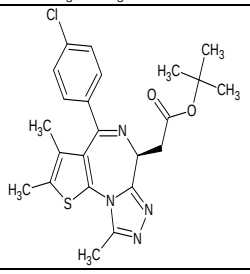
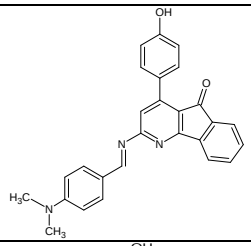
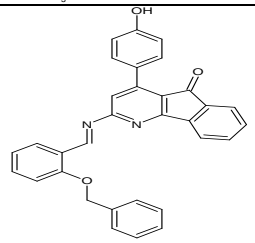
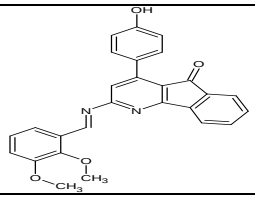
Compound code	Compound structure	Aqueous solubility Level	BBB Level	CYP2D6	Hepatotoxicity Level	PPB Level
2v		1	4	-1.39	-1.23	3.32
2w		0	4	-1.22	-2.11	2.57
2x		0	4	-1.42	-2.12	2.36
2y		0	4	-0.58	-1.71	2.52
JQ1		1	1	-5.26	-1.57	12.21

Table 3.5: Toxicity study of the designed pyrimidine compound.

Compound Code	Compound Structure	Aerobic Biodegradability probability	Aerobic Biodegradability prediction	Rat Inhalation LC_{50}	Rat Maximum Tolerated Dose feed
2a		0.03	Non-Degradable	3.09	0.17
2b		0.25	Non-Degradable	4.28	0.31
2c		0.22	Non-Degradable	9.96	0.19

3.5 Virtual toxicity studies

TOPKAT predicts endpoint of toxicity based mostly on chemical structure of Accelrys drug discovery studio 3.5. Including NTP carcinogenicity (female Rat, Male Rat), Ames Mutagenicity, Rat Oral LD_{50}, Skin irritation as well as improvement of toxicity. The different model were computed as well as recorded that satisfied all of the validation criteria for the query compound which are show in the **Table 3.5 & 3.6** The mutagenicity predict the drug's potential to result in human cellular to mutate, that is grounded on Ames research carcinogenicity assay and estimate the compound potential to trigger standard human cell to get cancer, the toxicity scientific studies was carried out for the female and male rat to minimize the time as well as price in the clinical trial. The skin irritation test support the topical usage of specific compound predicted to be not toxic in case it ranges from **0 to 0.29,** between **0.3 to 0.69** the compound is actually indeterminate of course, if it ranges from **0.7** as well as one is actually deadly. If the discriminant score is actually negative after that causing cancer is actually zero or maybe non carcinogenicity in case, the discriminant score is actually good the probability that will get cancer is actually rather high

Table 3.5: Toxicity study of the designed pyrimidine compound.

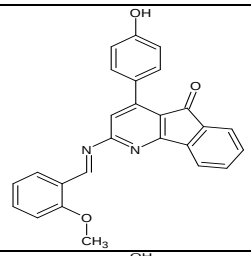
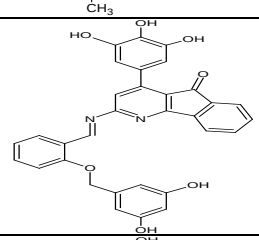
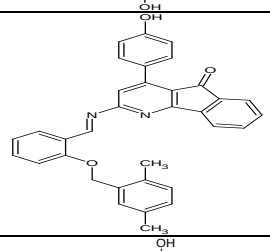
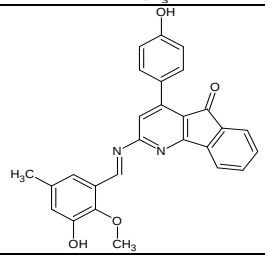
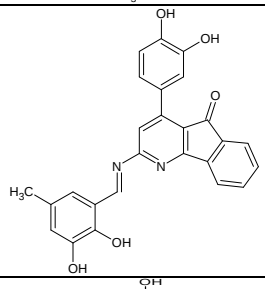
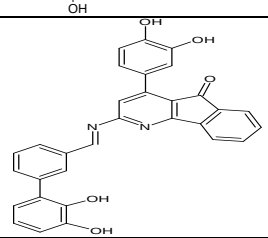
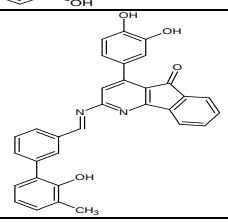
Compound Code	Compound Structure	Aerobic Biodegradability probability	Aerobic Biodegradability prediction	Rat Inhalation LC_{50}	Rat Maximum Tolerated Dose feed
2d		0.23	Non-Degradable	12.29	0.20
2f		0.21	Non-Degradable	0.70	0.77
2g		0.15	Non-Degradable	8.21	0.24
2h		0.13	Non-Degradable	8.74	0.25
2i		0.11	Non-Degradable	4.22	1.21
2j		0.19	Non-Degradable	1.70	1.33
2k		0.16	Non-Degradable	5.95	0.72

Table 3.5: Toxicity study of the designed pyrimidine compound.

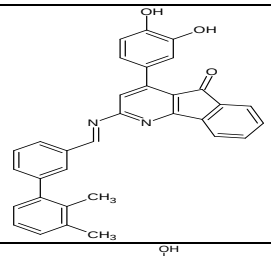
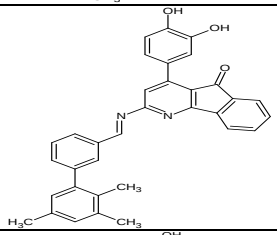
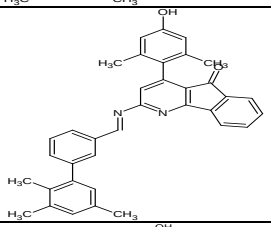
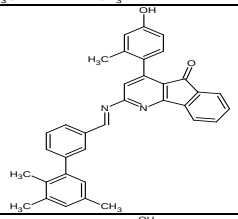
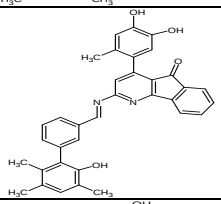
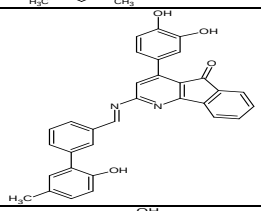
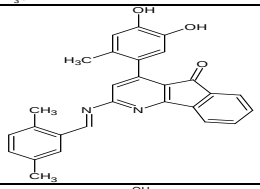
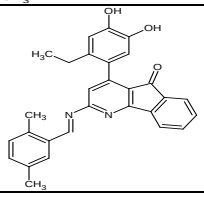
Compound Code	Compound Structure	Aerobic Biodegradability probability	Aerobic Biodegradability prediction	Rat Inhalation LC_{50}	Rat Maximum Tolerated Dose feed
2l		0.11	Non-Degradable	8.89	0.45
2m		0.09	Non-Degradable	8.35	0.43
2p		0.11	Non-Degradable	11.7155	0.26
2q		0.09	Non-Degradable	12.4798	0.27
2r		0.10	Non-Degradable	4.92215	0.61
2s		0.142104	Non-Degradable	5.95247	0.726415
2t		0.17	Non-Degradable	9.42	0.48
2u		0.12	Non-Degradable	16.20	0.53

Table 3.5 : Toxicity study of the designed pyrimidine compound.

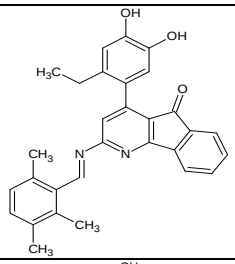
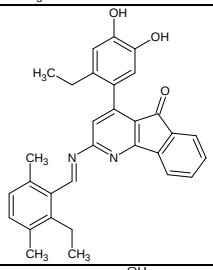
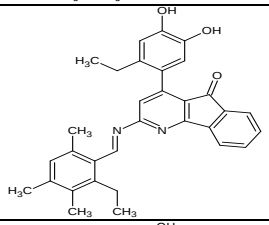
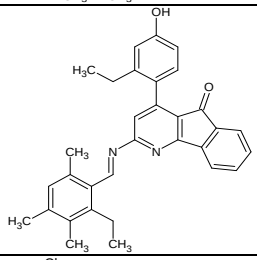
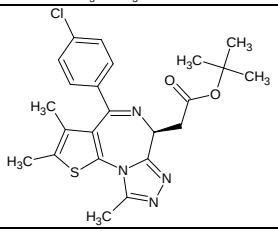
Compound Code	Compound Structure	Aerobic Biodegradability probability	Aerobic Biodegradability prediction	Rat Inhalation LC_{50}	Rat Maximum Tolerated Dose feed
2v		0.11	Non-Degradable	15.27	0.51
2w		0.12	Non-Degradable	14.29	0.54
2x		0.36	Non-Degradable	13.44	0.51
2y		0.09	Non-Degradable	21.35	0.34
JQ1		0.09	Non-Degradable	1.12	0.01

Table 3.6: Toxicity study of the designed pyrimidine compound

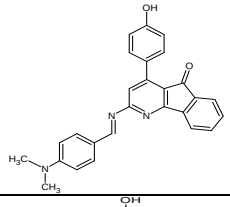
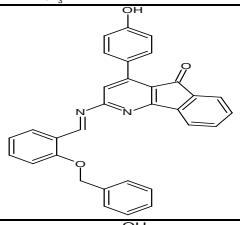
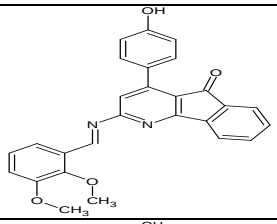
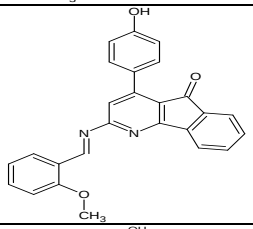
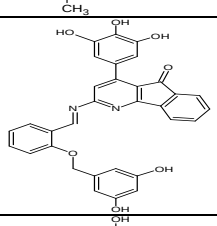
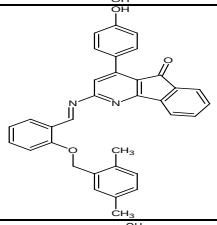
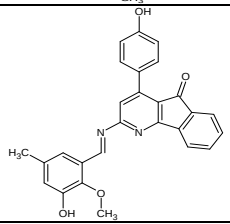
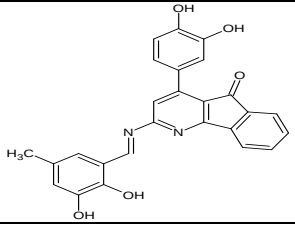
Compound Code	Compound structure	Ames Mutagenicity	Rat oral LD_{50}	Skin irritation	Ocular irritation
2a		6.06	0.55	None	Mild
2b		2.35	1.53	None	Mild
2c		2.90	1.04	None	Mild
2d		4.07	1.01	None	Mild
2f		1.24	1.86	None	Mild
2g		2.38	3.98	None	Mild
2h		1.97	1.67	None	Mild
2i		4.14	3.28	None	Mild

Table 3.6 : Toxicity study of the designed pyrimidine compound.

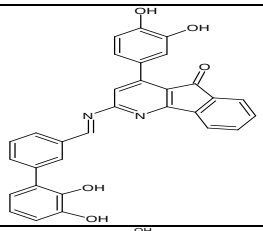
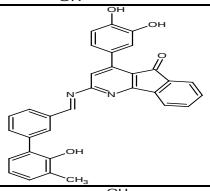
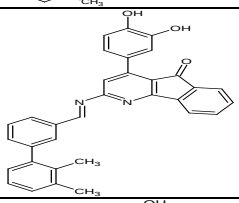
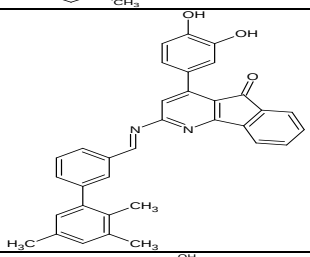
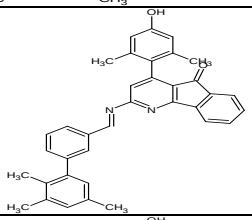
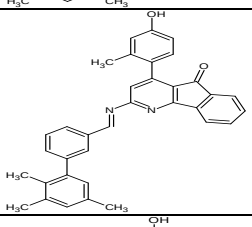
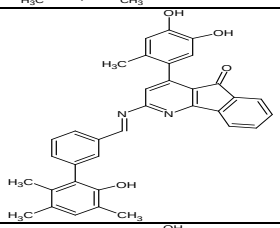
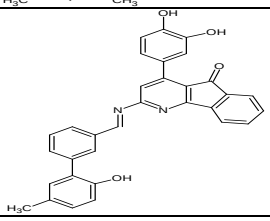
Compound Code	Compound structure	Ames Mutagenicity	Rat oral LD_{50}	Skin irritation	Ocular irritation
2j		4.21	1.60	None	Mild
2k		3.75	0.84	None	Mild
2l		4.31	0.67	None	Mild
2m		4.21	1.11	None	Mild
2p		3.05	0.55	None	Mild
2q		2.53	0.89	None	Mild
2r		3.37	0.68	None	Mild
2s		4.63	2.25	None	Mild

Table 3.6: Toxicity study of the designed pyrimidine compound

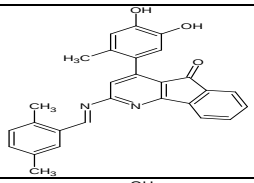
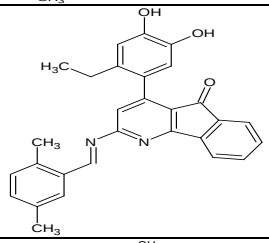
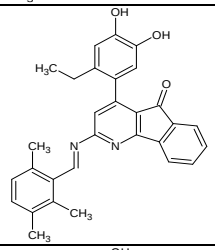
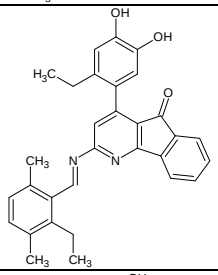
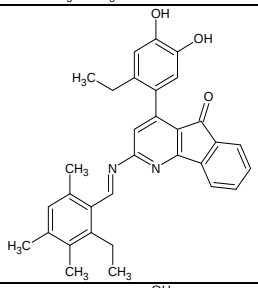
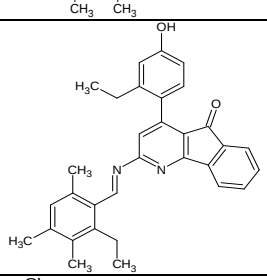
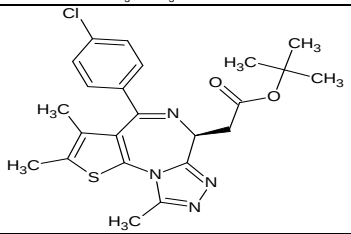
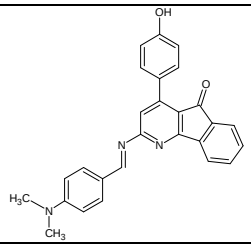
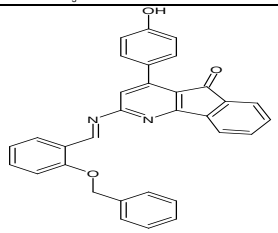
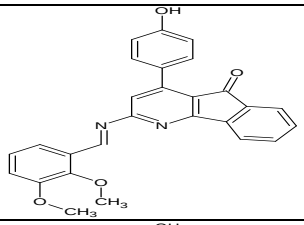
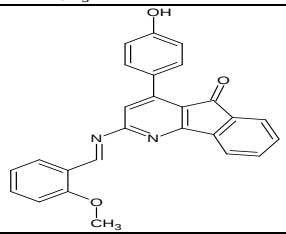
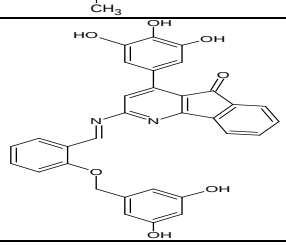
Compound Code	Compound structure	Ames Mutagenicity	Rat oral LD_{50}	Skin irritation	Ocular irritation
2t		1.62	0.94	None	Mild
2u		1.32	0.72	None	Mild
2v		2.10	0.32	None	Mild
2w		1.52	0.29	None	Mild
2x		1.24	0.40	None	Mild
2y		0.97	0.53	None	Mild
JQ1		-16.80	0.26	None	Mild

Table 3.7 drug likeness of the designed pyrimidine compound

Compound code	Compound Structure	No of H Bond donor	No of H Bond acceptor	A log Å	Molecular Weight	Molecular Fractional polar Surface areas
2a		1	5	5.77	419.47	0.16
2b		2	4	7.07	483.53	0.16
2c		2	5	5.47	437.46	0.20
2d		2	4	5.49	407.44	0.19
2f		5	8	5.40	546.52	0.31

3.7 Drug Likeness

The pyrimidine derivative compound show significant amount of hydrogen bond acceptor and donor. The hydrogen bond donor ranges from 0 to 5 whereas acceptor having 3 to 8. The compound had been developed to improve the binding with the receptor by ways of hydrogen bonding, all of the pyrimidine derivative follow the Lipinski rule of 5 as well as raises the drug likeness properties which are actually mention in **Table 3.7** polar surface areas have been taken into consideration of drug to permeate by cell membrane. All pyrimidine

derivatives compound are actually in the permissible limit and getting no bioavailability issue.

Table 3.7 drug likeness of the designed pyrimidine compound

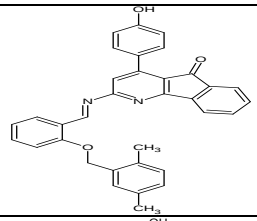
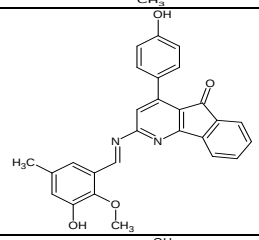
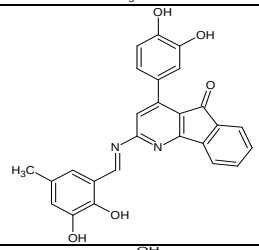
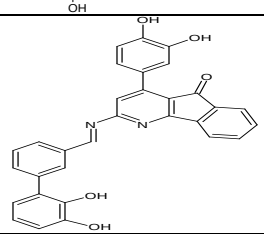
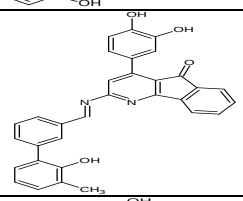
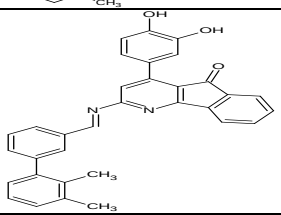
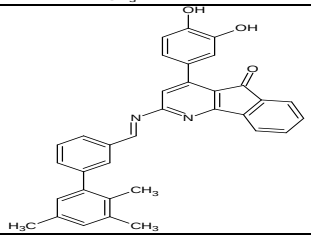
Compound code	Compound Structure	No of H Bond donor	No of H Bond acceptor	A log Å	Molecular Weight	Molecular Fractional polar Surface areas
2g		2	4	8.04	511.59	0.15
2h		3	5	5.73	437.46	0.23
2i		5	6	5.26	439.43	0.30
2j		5	6	6.30	501.50	0.27
2k		4	5	7.02	499.53	0.22
2l		3	4	7.75	497.56	0.18
2m		3	4	8.24	511.51	0.17

Table 3.7 drug likeness of the designed pyrimidine compound

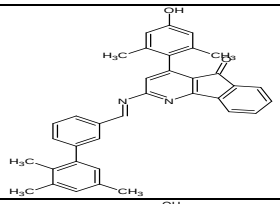
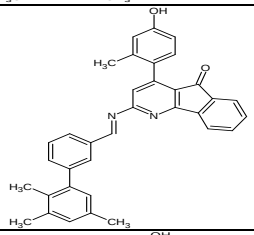
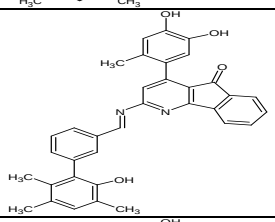
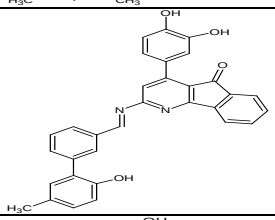
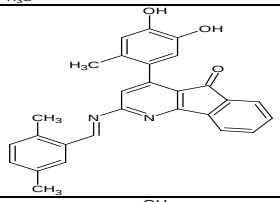
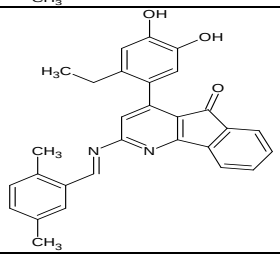
Compound code	Compound Structure	No of H Bond donor	No of H Bond acceptor	A log Å	Molecular Weight	Molecular Fractional polar Surface areas
2p		2	3	9.45	523.64	0.12
2q		2	3	8.97	509.61	0.13
2r		4	5	8.48	541.61	0.20
2s		4	5	7.02	499.53	0.22
2t		3	4	6.72	435.49	0.20
2u		3	4	7.18	449.52	0.19

Table 3.7 drug likeness of the designed pyrimidine compound

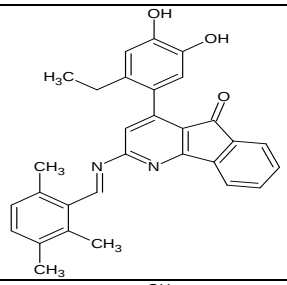
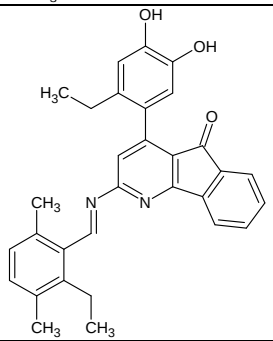
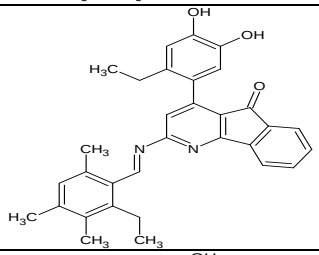
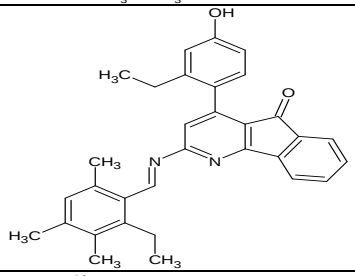
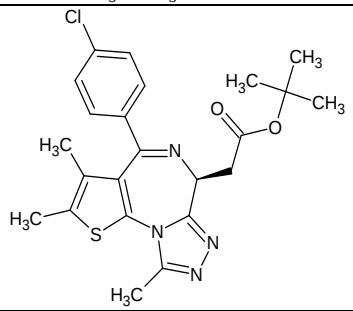
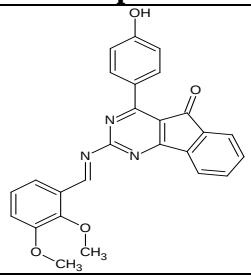
Compound code	Compound Structure	No of H Bond donor	No of H Bond acceptor	A log Å	Molecular Weight	Molecular Fractional polar Surface areas
2v		3	4	7.66	463.54	0.18
2w		3	4	8.12	477.57	0.17
2x		3	4	8.61	491.61	0.17
2y		2	3	8.85	475.60	0.13
JQ1		0	5	4.95	456.98	0.21

Table 3.8: Physicochemical parameter of synthesized pyrimidine derivative compound

Serial No.	Compound	Mol. Weight	Melting point (˚C)*	Yield (%)	RF Value**
1.		437.45	180-182	84.07	0.878

Physicochemical parameter

*Melting point are uncorrected

** Solvent system-pet ether: ethyl acetate 1:1

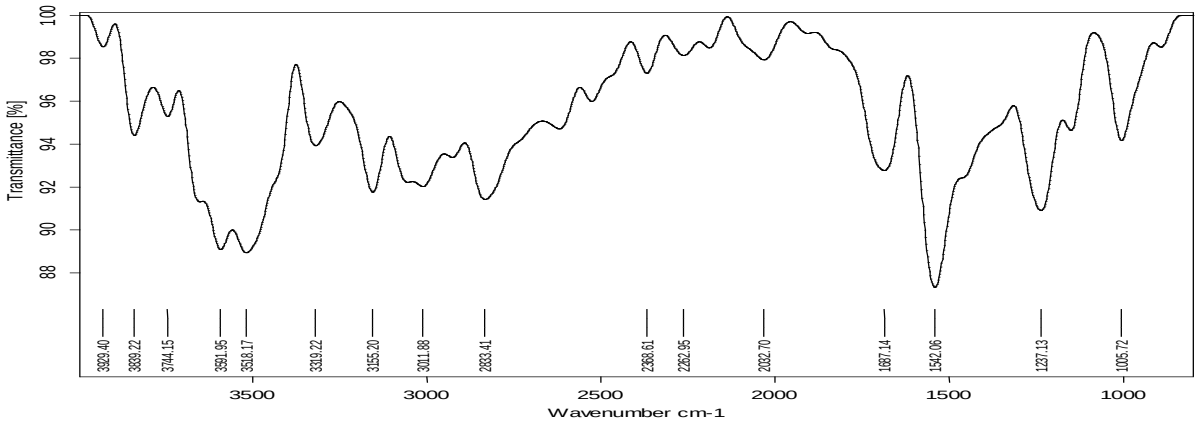
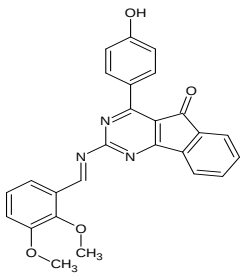


Fig. 9 IR spectra of 2-{(2, 3-dimethoxy benzylidene) amino)-4-(4-hydroxyphenyl)-5H-indeno {1, 2-d} pyrimidine -5-one



IR (cm^{-1}) V: 1693 (C=O), Stretch), 1569.79 (C=N, Stretch), 3030.59 (CH, Stretch), 1257.75 (C-O-C, bend), 3621.63 (OH), Stretch)

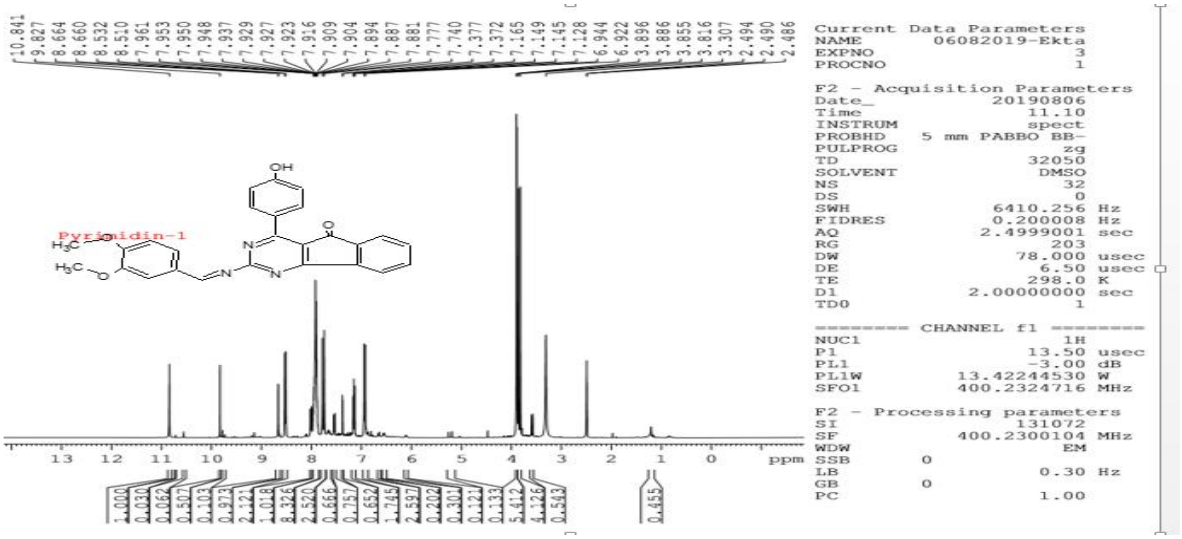


Fig. 10 ^{1}H NMR spectra of 2-{(2, 3-dimethoxy benzylidene) amino)-4-(4-hydroxyphenyl)-5H-indeno {1, 2-d} pyrimidine -5-one

^{1}H NMR (400 MHz, $CDCl_3$): 3.88 (S, 3H, OCH_3), 9.827 (S, 1H, N=CH), 8.531-8.510 (d, 2H, Ar-H), 7.77-8.020 (m, 10H, Ar-H)

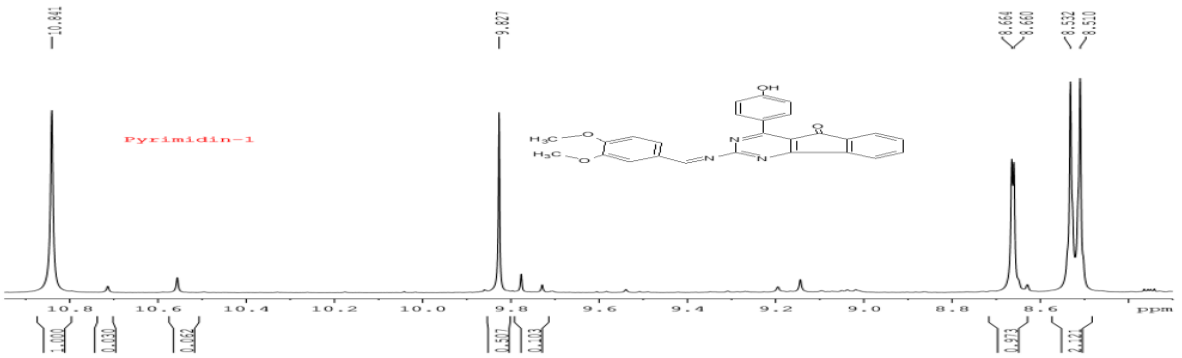


Fig. 11 ^{1}H NMR spectra of 2-{(2, 3-dimethoxy benzylidene) amino)-4-(4-hydroxyphenyl)-5H-indeno {1, 2-d} pyrimidine -5-one.

^{1}H NMR (400 MHz, $CDCl_3$): 3.88 (S, 3H, OCH_3), 9.827 (S, 1H, N=CH), 8.531-8.510 (d, 2H, Ar-H), 7.77-8.020 (m, 10H, Ar-H)

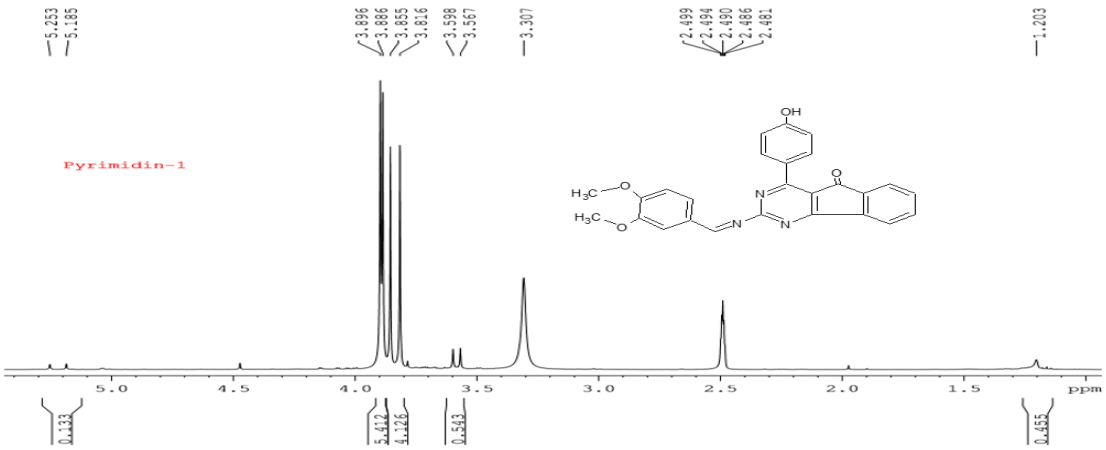


Fig. 12[1]H NMR spectra of 2-{(2, 3-dimethoxy benzylidene) amino)-4-(4-hydroxyphenyl)-5H-indeno {1, 2-d} pyrimidine -5-one.

^{1}H NMR (400 MHz, $CDCl_3$): 3.88 (S, 3H, OCH_3), 9.827 (S, 1H, N=CH), 8.531-8.510 (d, 2H, Ar-H), 7.77-8.020 (m, 10H, Ar-H)

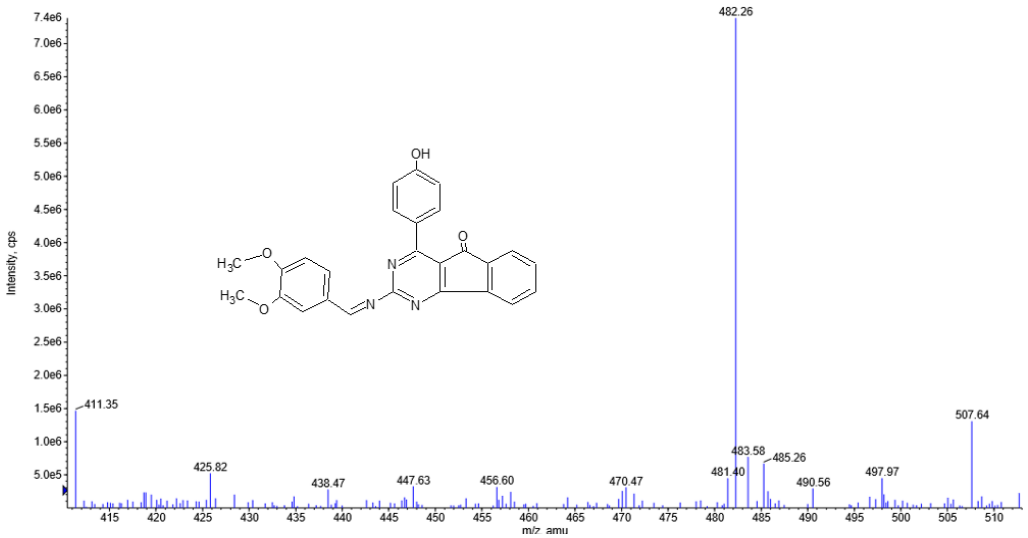


Fig. 13 Mass spectra of 2-{(2, 3-dimethoxy benzylidene) amino)-4-(4-hydroxyphenyl)-5H-indeno {1, 2-d} pyrimidine -5-one

MS m/z (%) 437.44 ($C_{26}H_{19}N_30_4$)

3.9 The *in vitro* model on leukemia (HL-60) cell line

Table 3.9: Effect of different concentration of synthesized pyrimidine derivative molecule and standard 5 FU drug on leukemia (HL-60) cell line are given in Table 3.9 and Fig. 13 & 14.

Drugs	% inhibition at 25 µg	% inhibition at 50 µg	% inhibition at 75 µg	% inhibition at 100 µg
Synthesized pyrimidine derivative compound	16.86 ± 0.41***	32.58 ± 0.43***	43.70 ± 0.32***	54.99 ± 0.83***
Standard 5 FU	7.01 ± 0.90***	23.6 ± 0.30***	33.7 ± 0.33***	51.1 ± 0.89***

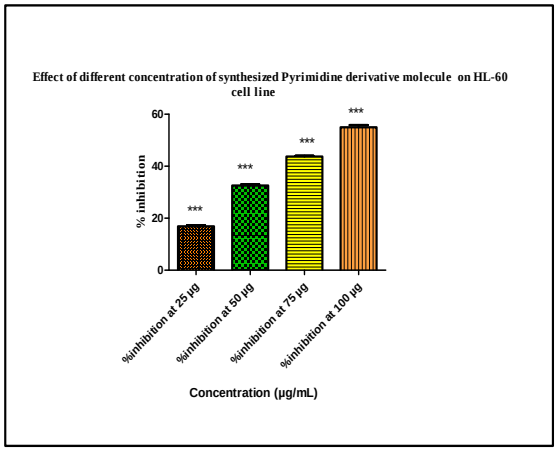


Fig. 14

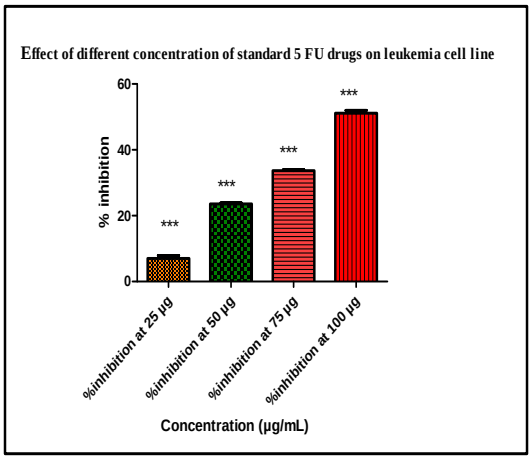


Fig. 15

Effect of synthesized compound 2c and standard 5-FU are represented in bar diagram in fig. 14 &15.

n=3 in each concentration, the values are expressed as Mean ± SEM Where, ***P<0.05(*), P<0.01(**), P<0.001(***)***, were taken as significant when compared with Standard drug. Data were analyzed by one-way ANOVA followed by Dunnett's test.

The result shows that the synthesized pyrimidine derivative compound inhibits more leukemia (HL-60) cancer cell line as compared to standard 5 FU drug with increase concentration as shown in **Table 3.9 and fig. 14 & 15** and ***P<0.001(***)***

Cytotoxicity study by MTT assay

Ligand pyrimidine derivative was assay against selected cancer cell lines such as **HL-60** (human leukemia cell line) to determine its efficiency to inhibit the human leukemia cancer

cells viability in the presence of different concentrations of the inhibitor **(25 µg, 50 µg, 75 µg, 100 µg)**

Table 3.9.1: MTT assay shows the percentage cell inhibition of pyrimidine (Test) in different concentration.

Concentration (µg)	% inhibition of Test (Pyrimidine)	IC_{50} µMol
25	16.86 ± 0.41***	83
50	32.58 ± 0.43***	
75	43.70 ± 0.32***	
100	54.99 ± 0.83***	

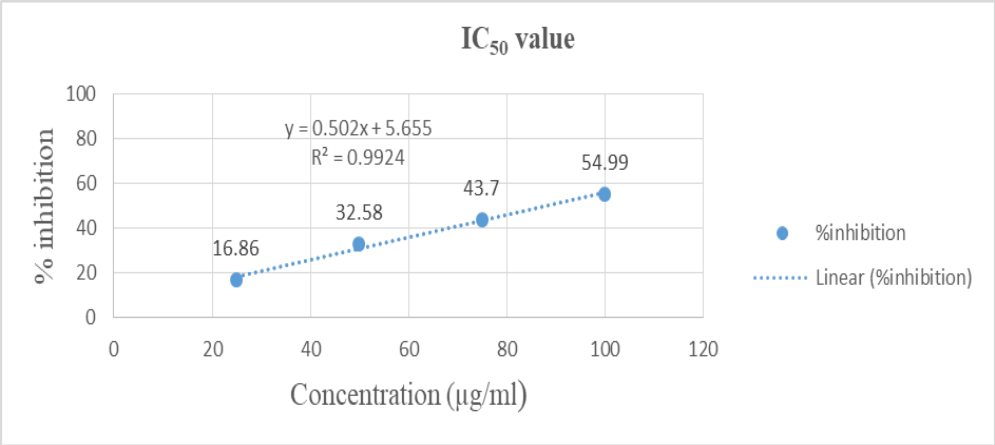


Fig. 16 IC_{50} plot pyrimidine (Test).

Highest activity is 54.99 % obtained at the concentration of 100 µg
Lowest activity is 16.86 % obtained at the concentration of 25 µg
IC_{50} = 83 µMol

Table 3.9.2: MTT assay shows the percentage cell inhibition of 5-FU (Standard) in different concentration

Concentration (µg)	% Inhibition of Standard (5 FU)	IC_{50} µMol
25	7.01 ± 0.90***	100
50	23.6 ± 0.30***	
75	33.7 ± 0.33***	
100	51.1 ± 0.89***	

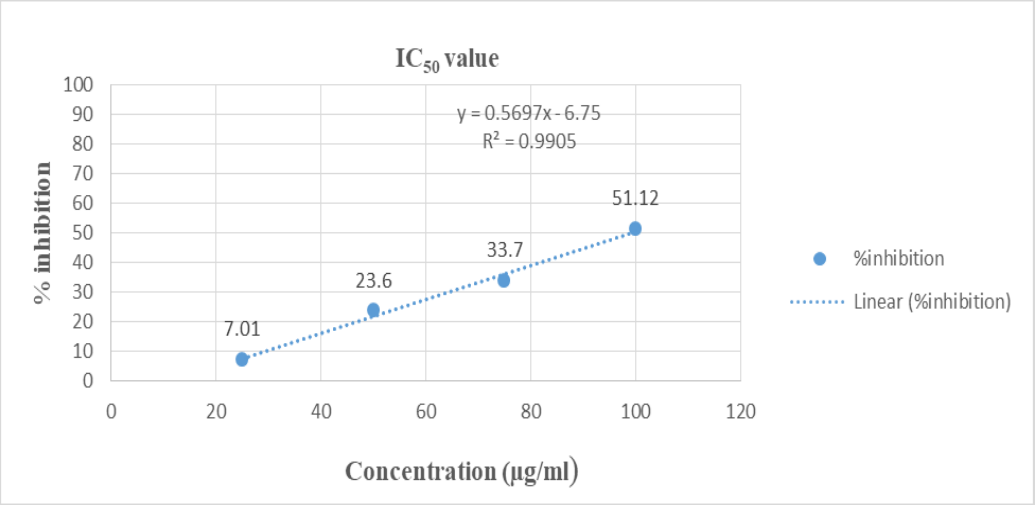


Fig. 17 IC_{50} plot of standard (5 FU).

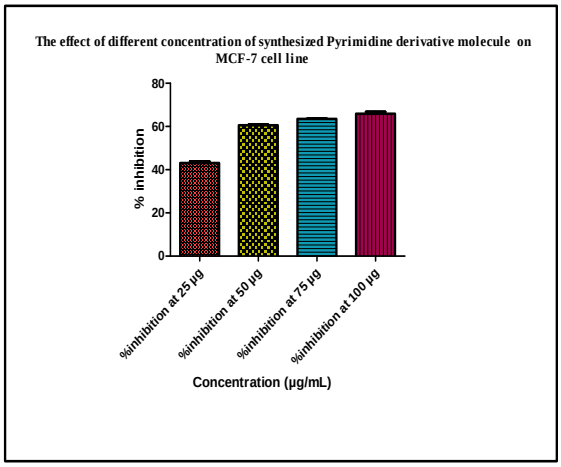
Highest activity is 51.1% obtained at the concentration of 100 µg
Lowest activity is 7.01% obtained at the concentration of 25 µg
IC_{50} = 100 µMol

3.9.3 The *in vitro* model Breast cancer (MCF-7) cell line

Table 3.9.3: Effect of different concentration of synthesized pyrimidine derivative compound and standard 5 FU drug on Breast cancer (MCF-7) cell line are given in Table 3.9.3 and Fig. 18 & 19

Drugs	% inhibition at 25 µg	% inhibition at 50 µg	% inhibition at 75 µg	% inhibition at 100 µg
Pyrimidine derivative	43.1 ± 0.77***	60.5 ± 0.28***	63.5 ± 0.08***	65.9 ± 0.84***
Standard 5 FU	7.39 ± 0.02***	16.3 ± 0.03***	24.9 ± 0.41***	60.2 ± 0.55***

n= 3 in each concentration, the values are expressed as Mean ± SEM Where, ***P<0.05(*), P<0.01(**), P<0.001(***)***, were taken as significant when compared with Standard drug. Data were analyzed by one-way ANOVA followed by Dunnett's test.



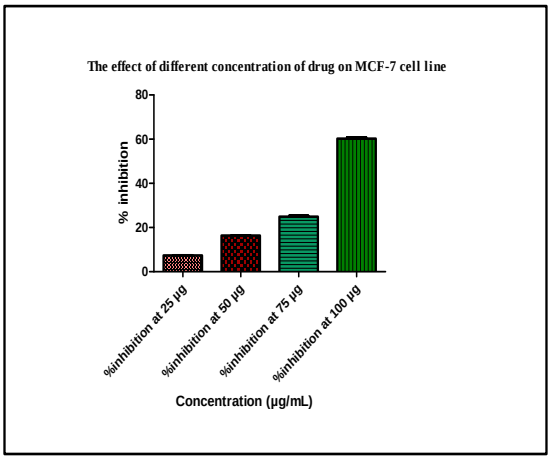


Fig. 18 **Fig. 19**

Effect of synthesized compound 2c and standard 5-FU are shown in bar diagram are given in fig. 18 & 19.

The result shows that the synthesized Pyrimidine derivative molecule inhibits more breast cancer (MCF-7) cell line as compared to standard 5 FU drug with increase concentration as shown in **Fig. 18 & 19** and **Table 3.9.3** ***P<0.001(***)***

Cytotoxicity study by MTT assay

Ligand pyrimidine derivative was assay against selected cancer cell lines such as **MCF-7** (Breast cancer cell line) to determine its efficiency to inhibit the breast cancer cells viability in the presence of different concentrations of the inhibitor **(25 µg, 50 µg, 75 µg, 100 µg)**

Table 3.9.4: MTT assay shows the percentage cell inhibition of pyrimidine (Test) in different concentration.

Concentration (µg)	% inhibition of Test (Pyrimidine)	IC_{50} µMol
25	43.1 ± 0.77	40
50	60.5 ± 0.28	
75	63.5 ± 0.08	
100	65.9 ± 0.84	

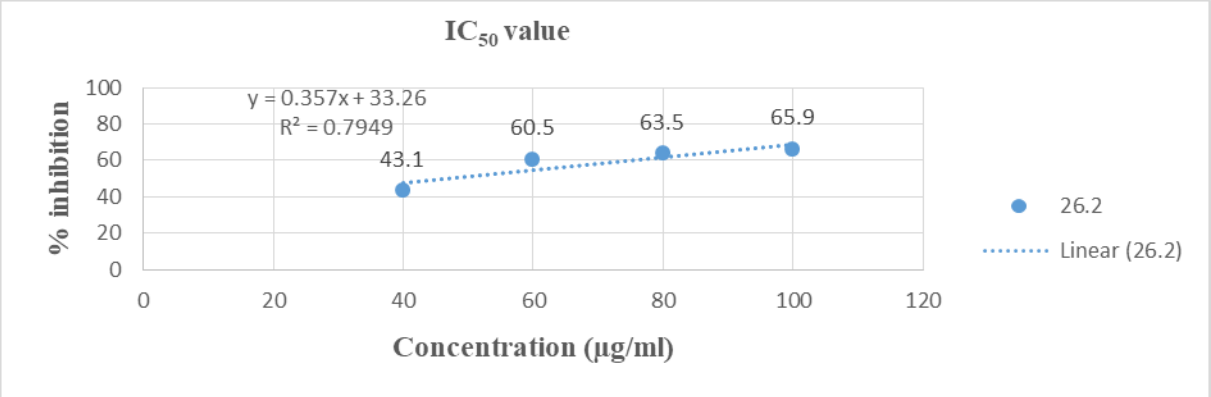


Fig. 20 IC_{50} plot pyrimidine (Test).

Highest activity is 65.9 % obtained at the concentration of 100 µg

Lowest activity is 43.1 % obtained at the concentration of 25 µg

IC_{50} = 40 µMol

Table 3.9.5: MTT assay shows the percentage cell inhibition of Imantinib Mesylate (Standard) in different concentration.

Concentration (µg)	% inhibition of Standard (Imantinib Mesylate)	IC_{50} µMol
25	7.39 ± 0.02***	100
50	16.3 ± 0.03***	
75	24.9 ± 0.41***	
100	60.2 ± 0.55***	

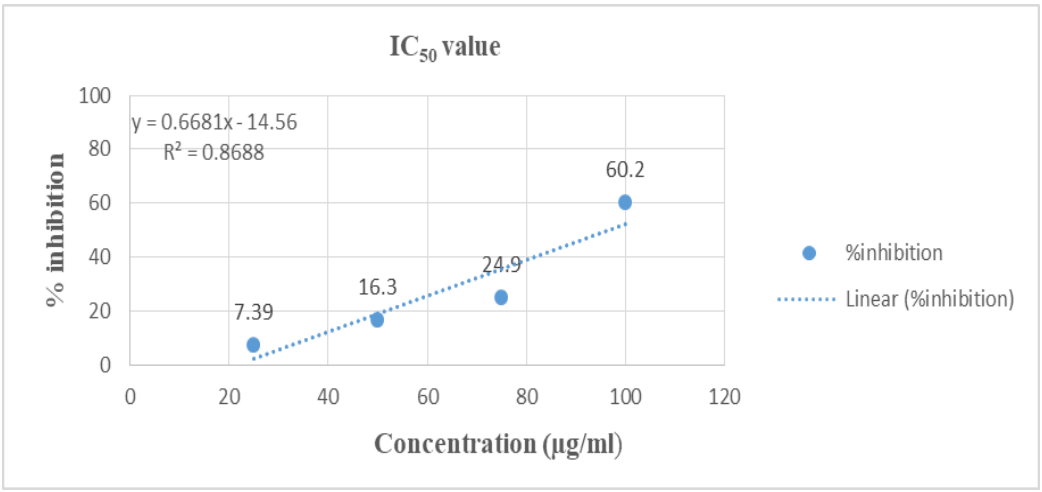


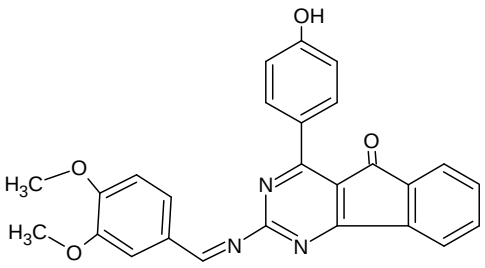
Fig. 21 IC_{50} plot of standard (Imantinib Mesylate)

Highest activity is 60.2% obtained at the concentration of 100 µg

Lowest activity is 7.39% obtained at the concentration of 25 µg

IC_{50} = 100 µMol

Table 3.9.6: Different cancer cell line with IC_{50} value.

Compound Structure	IC_{50} µMol					
	HL-60		Hela		MCF-7	
	Test	5 FU	Test	5 FU	Test	Imantinib Mesylate
	83	100	104	83	40	100
	HCT-116		A431			
	Test	Imantinib Mesylate	Test	Imantinib Mesylate		
	44	10	44	10		

n=3 in each concentration, the values are expressed as Mean ± SEM Where, ***P<0.05(*), P<0.01(**), P<0.001(***)***, were taken as significant when compared with Standard drug. Data were analyzed by one-way ANOVA followed by Dunnett's test.

3.9.7 The *in vitro* model on breast cancer cell line (MCF-7)

Table 3.9.7: Effect of concentration of synthesized pyrimidine derivative molecule and standard JQ1 inhibitor on breast cancer cell line are given in Table 3.9.7 and Fig. 22.

Drugs	% BRD4 inhibition at 25 µM in MCF-7
Synthesized pyrimidine derivative compound	31.73 ± 1.28
Standard JQ1	67.44 ± 1.03

The result shows that the synthesized pyrimidine derivative compound inhibits less breast cancer cell line as compared to standard JQ1 inhibitor at **25 µM** as shown in **Table 3.9.7 & fig. 22.**

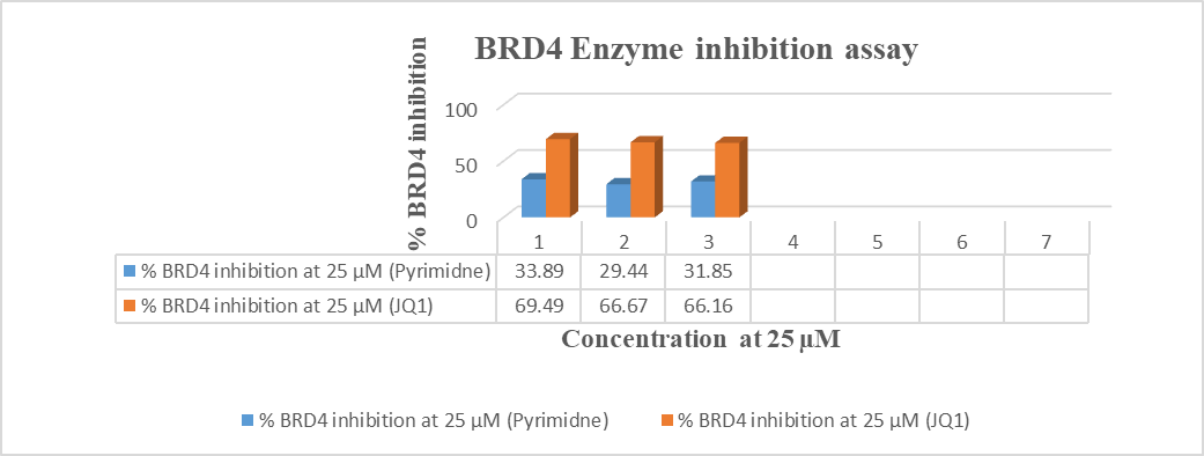


Fig. 21 % BRD4 inhibition at 22 µM in MCF-7.

3.9.8 *In vivo* EAC model

Table 3.9.8: The effect of drugs on Hgb, RBCs, and WBCs.

Groups	Hgb (g/dl)	RBC 1x10^6 /mm^3	WBC 1x10^3/mm^3
Cancer Control	4.7 ± 0.30	2.05 ± 0.11	30.48 ± 0.45
Synthesized Pyrimidine derivative compound (200 mg/kg)	10.4 ± 0.08 ***	5.67 ± 0.15***	17.33 ± 0.39***
5 FU (20 mg/kg)	11 ± 0.35 ***	5.99 ± 0.154***	15.63 ± 1.46***

n= 8 mice in each group. Values are expressed as Mean ± SEM Where, ***P<0.05(*), P<0.01(**), P<0.001(***)***, were taken as significant when compared with control. Data were analyzed by one-way ANOVA followed by Dunnett's test.

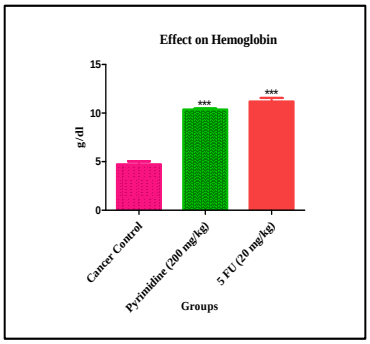


Fig. 23 Effect of drugs on Hemoglobin

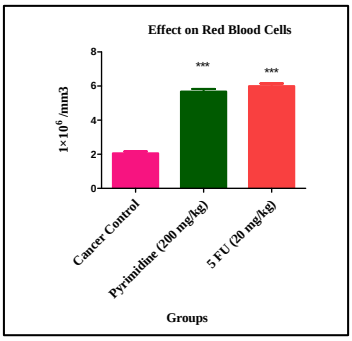


fig. 24 Effect of drugs on RBCs

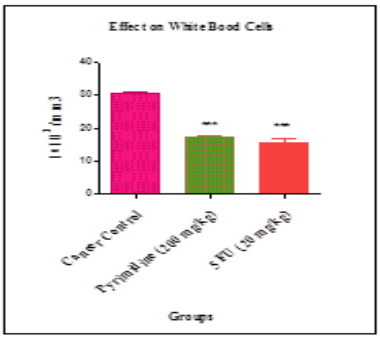


fig. 25 Effect of drugs on WBCs

The effect of synthesized pyrimidine derivative compound (200 mg/kg) and standard 5 FU (20 mg/kg) on Hemoglobin, Red blood cells, white blood cell in EAC induced mice are given in **Table 3.9.8** and **Fig. 23, 24 and 25.**

Fig. 23 result shown that level of Hgb was decreased in EAC cancer control mice i.e., (4.7 ± 0.30 g/dL) when compared to synthesized pyrimidine derivative compound (200 mg/kg) (10.4 ± 0.08 g/dL) ***P<0.001(***)*** and standard 5 FU (20 mg/kg) (11 ± 0.35 g/dL) ***P<0.001(***)*** **Fig. 24** Treatment with synthesized pyrimidine derivative compound (200

mg/kg) showed an extremely significant ***(P<0.001)*** increase (5.67 ± 0.15) $1x10^6$ /mm^3in RBC count as compared to EAC cancer control group (2.05 ± 0.11) $1x10^6$ /mm^3, while significant ***(P<0.001)*** increase (5.99 ± 0.154) $1x10^6$ /mm^3 in RBC count on administration of standard of 5 FU (20 mg/kg).

Fig. 25 result shown that level of WBC was increase in EAC cancer control mice i.e., (30.48 ± 0.45) $1x10^3$/mm^3 when compared to synthesized pyrimidine derivative compound (200 mg/kg) (17.33 ± 0.39) $1x10^3$/mm^3 ***P<0.001(***)*** and standard 5 FU (20 mg/kg) (15.63 ± 1.46) $1x10^3$/mm^3 ***P<0.001(***)***

Table 3.9.9: Increase in life span.

Groups	MST (days)	%ILS
Cancer Control	15 ± 0.32	0
Synthesized pyrimidine derivative compound (200 mg/kg)	23 ± 0.57***	87.5
5 FU (20 mg/kg)	22 ± 0.53***	87.5

The effect of synthesized pyrimidine derivative compound (200 mg/kg) and standard 5 FU (20 mg/kg) on Mean Survival Time (MST) and percentage increase in life span (% ILS) in EAC induced mice are given in **Table 3.9.9** and **Fig. 26**

n= 8 mice in each group. Values are expressed as Mean ± SEM Where, ***P<0.05(*), P<0.01(**), P<0.001(***)***, were taken as significant when compared with control. Data were analyzed by one-way ANOVA followed by Dunnett's test

The result showed that in EAC cancer control mice the Mean survival time was (15 ± 0.32) days. Whereas, it was significantly increased on treatment with test synthesized pyrimidine derivative compound (200 mg/kg) by (87.5 %) (23 ± 0.57) days ***(P<0.001)*** Where, comparison on treatment with standard drug 5 FU (20 mg/kg) increased the life span by (87.5 %) and increased the mean survival time significantly (22 ± 0.53) days (***P<0.001).***

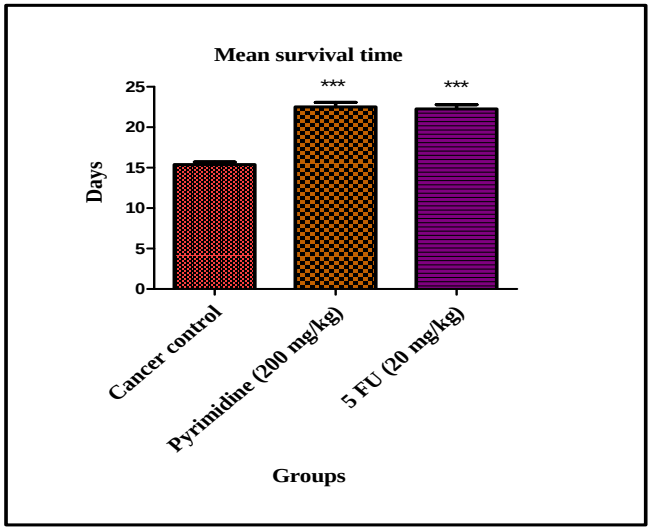


Fig. 26 Mean survival time.

Effect of synthesized compound 2c and standard 5 FU on mean survival time are given below

Table 3.9.9.1: Effect of drugs on body weight of mice

Animals	Body weight in (g) (Cancer control group)				Body weight in (g) (Synthesized pyrimidine derivative compound group)				Body weight in (g) (5 FU group)			
	Days				Days				Days			
	0	7	14	21	0	7	14	21	0	7	14	21
1	37.2	40.0	42.0	D	32.4	23.6	22.1	21.1	36.2	35.2	34.0	32.0
2	38.4	41.2	42.3	D	31.3	30.8	28.2	27.4	40.3	38.4	36.4	34.2
3	33.2	38.2	40.0	D	28.2	23.5	21.2	D	40.1	38.6	36.4	35.6
4	37.4	41.3	42.1	D	24.7	21.2	20.1	19.6	40.2	37.8	39.4	D
5	35.4	39.2	42.0	D	32.1	31.0	28.4	25.4	38.3	32.6	31.4	30.0
6	35.4	38.4	40.2	D	31.1	30.7	29.1	27.2	38.2	37.1	35.4	33.2
7	34.5	36.4	39.8	D	32.2	30.2	29.1	28.3	39.2	38.4	35.4	33.6
8	35.3	36.4	38.3	D	34.3	32.4	30.1	29.3	41.1	40.2	38.4	36.4

D → Death

Table 3.9.9.2: Effect of drugs on body weight of mice.

Groups	Change in body weight (g)			
	0 day	7 days	14 days	21 days
Cancer Control	35.9 ± 0.5 g	38.9 ± 0.6 g	40.8 ± 0.5 g	(death)
Synthesized pyrimidine derivative compound (200 mg/kg)	30.8 ± 1.0 g	28 ± 1.5 g ***	26.8 ± 1.3 g ***	23 ± 3.4 g ***
5 FU (20 mg/kg)	39.1 ± 0.5 g	37.3 ± 0.8g (ns)	35 ± 0.8 g **	29.1 ± 4.26g ***

The effect synthesized pyrimidine derivative compound (200 mg/kg) and standard 5 FU (20 mg/kg) on body weight in EAC induced mice are given in **Table 3.9.9.2** and **Fig. 27, 28, 29 and 30**

n= 8 mice in each group. Values are expressed as Mean ± SEM Where, ***P<0.05(*), P<0.01(**), P<0.001(***)***, were taken as significant when compared with control. Data were analyzed by one-way ANOVA followed by Dunnett's test.

Effect of synthesized pyrimidine derivative and standard 5-FU on body weight are represented in bar diagram of given **fig. 27, 28, 29 and 30**

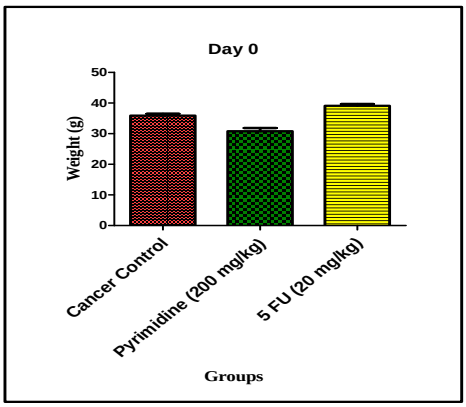


Fig. 27 Body weight analysis 0 day

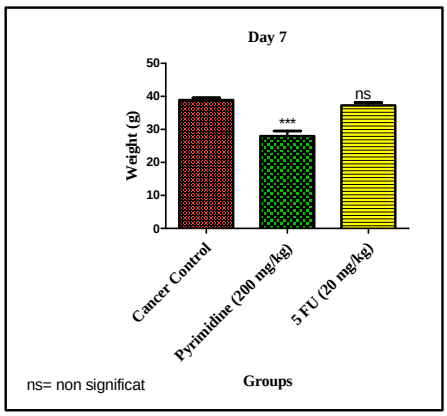


Fig. 28 Body weight analysis 7 days

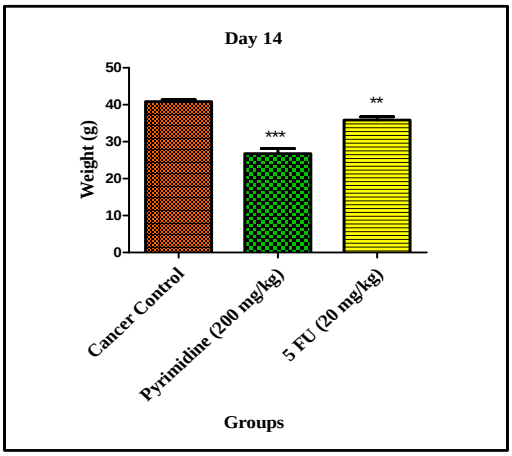


Fig. 29 Body weight analysis 14 days.

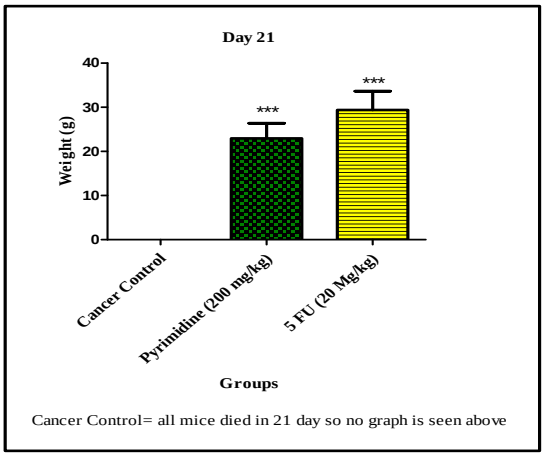


Fig. 30 Body weight analysis 21days.

The above **Table 16** and **Fig. 27, 28, 29 and 30** result show that animal were induce with EAC cancer cell in three group i.e.., cancer control group, test group and standard group. The body weight of cancer control mice increases with respect to day increases at 0 day (35.9 ± 0.5) g, 7 days (38.9 ± 0.6) g, 14 days (40.8 ± 0.5) g and 21 days (death of animals) whereas

the body weight get decrease in both test synthesized pyrimidine derivative compound (200 mg/kg), 0 day (30.8 ± 1.0) g, 7 days (28 ± 1.5) g, 14 days (26.8 ± 1.3) g and 21 days (23 ± 3.4) g and standard drug 5 FU (20 mg/kg), 0 day (39.1 ± 0.5) g, 7 days (37.3 ± 0.8) g, 14 days (35 ± 0.8) g and 21 days (29.1 ± 4.26) g on comparison with 0 day treatment.

Table 3.9.9.3: The effect of drugs on antioxidant parameter.

Groups		Cancer Control	Pyrimidine (200 mg/kg)	5 FU (20 mg/kg)
SOD (IU/mg protein)	Kidney	0.06±0.01	0.18±0.02 **	0.18±0.03 ***
	Liver	0.22±0.03	0.64±0.05 **	0.64±0.03 ***
GSH (mM/g)	Kidney	0.46±0.03	0.71±0.06 **	0.74±0.02 ***
	Liver	0.48±0.01	0.69±0.05 **	0.93±0.02 ***
Catalase (µM/mg protein)	Kidney	0.57±0.03	0.92±0.03 ***	0.86±0.04 ***
	Liver	0.56±0.02	0.80±0.01 ***	0.87±0.01 ***
Nitric oxide (mM/ml)	Kidney	0.54±0.01	0.77±0.01 ***	0.76±0.02 ***
	Liver	0.66±0.01	0.80±0.02 ***	0.82±0.01 ***
Total protein (mg/dL)	Kidney	2.32 ± 0.08	1.42 ± 0.06 ***	1.62 ± 0.12 ***
	Liver	2.21 ± 0.06	1.43 ± 0.08 ***	1.48 ± 0.09 ***

n = 6 mice in each group. Values are expressed as Mean ± SEM Where, P<0.05(*), P<0.01(**), P<0.001(***),

Were taken as significant when compared with control. Data were analyzed by one-way ANOVA followed by Dunnett's test.

Effect of synthesized pyrimidine derivative and standard 5-FU on kidney and liver are represented in bar diagram of given **fig. 31, 32, 33, 34, 35, 36, 37, 38, 39 and 40.**

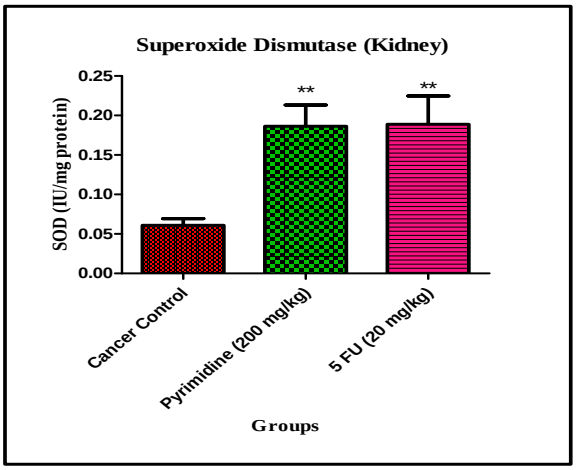


Fig. 31 Effect of drugs on Superoxide Dismutase.

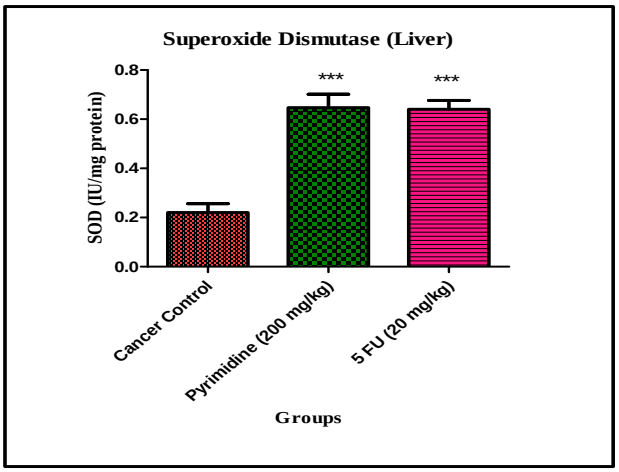


Fig. 32 Effect of drugs on Superoxide Dismutase.

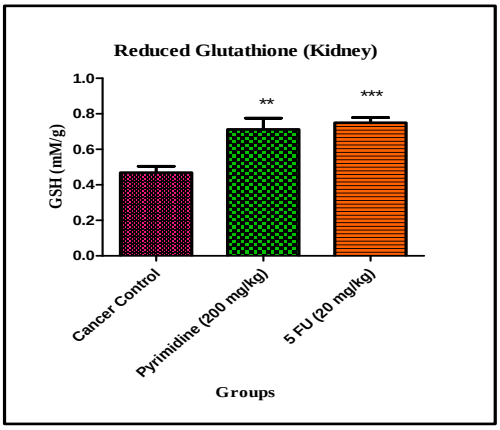


Fig. 33 Effect of drugs on Reduced Glutathione

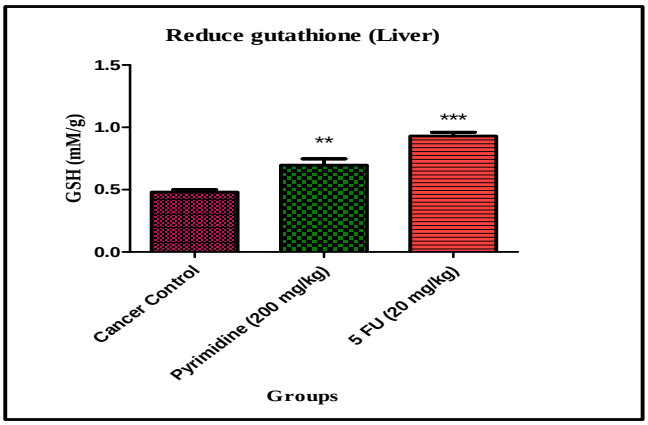


Fig. 34 Effect of drugs on Reduced Glutathione.

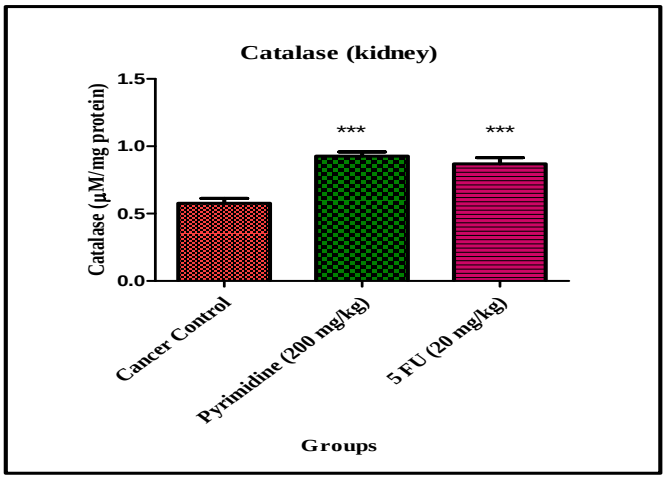


Fig. 35 Effect of drugs on Catalase

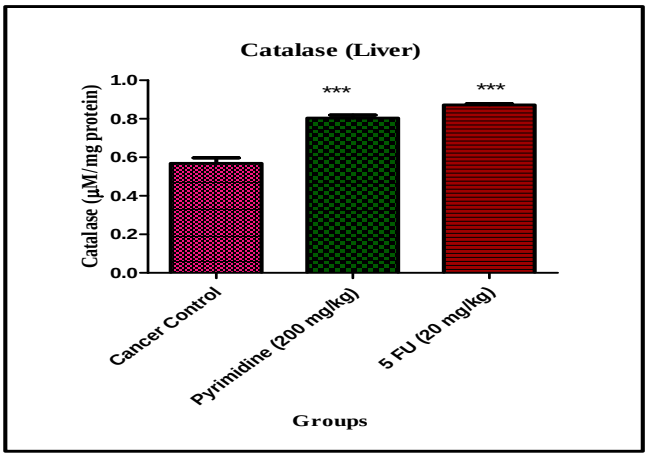


Fig. 36 Effect of drugs on Catalase

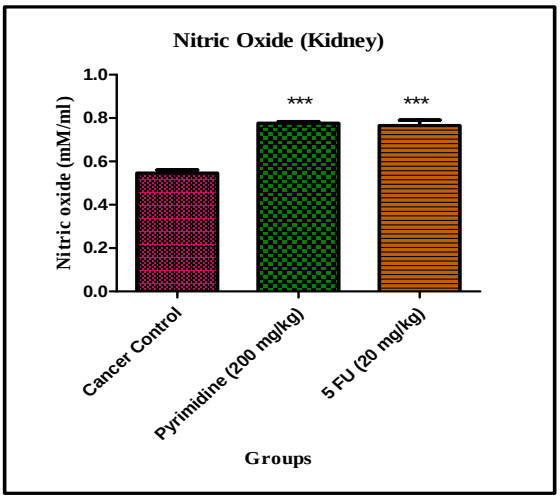


Fig. 37 Effect of drugs on Nitric Oxide.

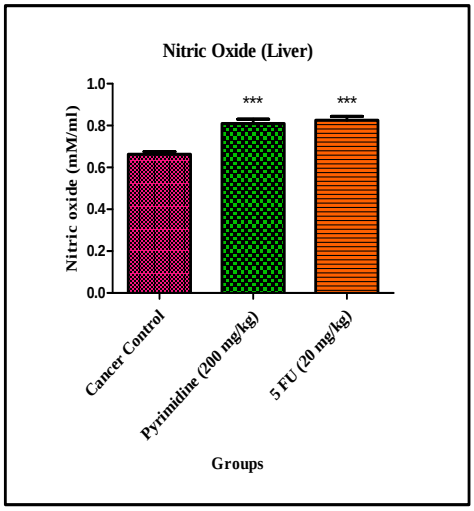


Fig. 38 Effect of drugs on Nitric Oxide

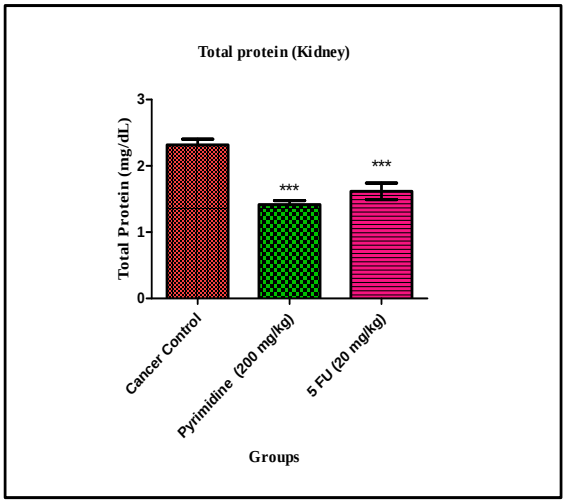


Fig. 39 Effect of drugs on total protein.

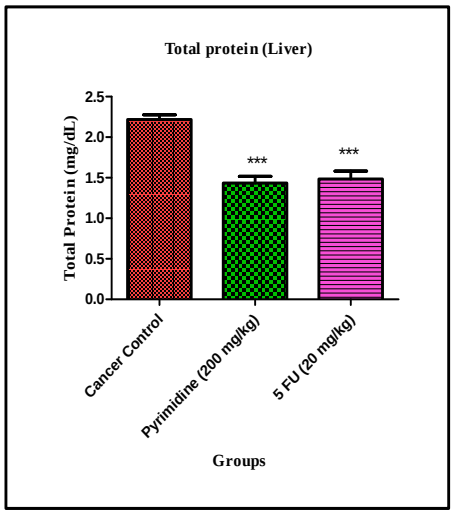


Fig. 40 Effect of drugs on total protein.

Experimental result represented in **Table 3.9.9.3 and fig. 31, 32, 33, 34, 35, 36, 37, 38, 39 and 40 for superoxide dismutase (SOD), Reduced Glutathione, Catalase, Nitric oxide activity and total protein**. The 24 mice were first induce with EAC cancer cell and divided into three group. the each group consist of 8 mice, the group which were divided named as cancer control group (0.9% normal saline), test group (200 mg/kg/p.o) and standard group (20 mg/kg/p.o) and the superoxide dismutase (SOD), Reduced Glutathione, Catalase, Nitric oxide activity and total protein were estimated and result found that The cancer control mice SOD contents were significantly decreases in both the kidney and liver whereas the SOD content get significantly increases in both the kidney and liver on treatment with test synthesized pyrimidine derivative compound (200 mg/kg) and standard drug 5 FU (20 mg/kg). The cancer control mice GSH level were significantly decreases in both the kidney

and liver whereas the GSH contents get significantly increases in both the kidney and liver on treatment with test synthesized pyrimidine derivative compound (200 mg/kg) and standard drug 5 FU (20 mg/kg), The cancer control mice **catalase** level were significantly decreases in both the kidney and liver whereas the **catalase** level get significantly increases in both the kidney and liver on treatment with test synthesized pyrimidine derivative compound (200 mg/kg) and standard drug 5 FU (20 mg/kg), the cancer control mice **nitric oxide** level were significantly decreases in both the kidney and liver whereas the **nitric oxide** level get significantly increases in both the kidney and liver on treatment with test synthesized pyrimidine derivative compound (200 mg/kg) and standard drug 5 FU (20 mg/kg), The cancer control mice level were significantly increase in both the kidney and liver whereas on treatment with test synthesized pyrimidine derivative compound (200 mg/kg) and standard drug 5 FU (20 mg/kg), **total protein** level get significantly decreases in both the kidney and liver

4. DISCUSSION

In the current study, we investigated that the synthesized pyrimidine compound shows inhibition toward bromodomain protein, pyrimidine compound consist of six membered heterocyclic ring that constitutes numerous part of nucleic acid and utilized as a pharmacophore for the synthesis of countless drugs like anticancer, antibacterial and antiviral agents. (Prachayasittikul S, 2011) pyrimidine based anticancer drug have been developed based on structural modification of core structures (i.e., substitution with rings and moieties, conjugation with some other compounds, and control with metallic ions). The pyrimidine derivative molecules shows ability as privileged scaffolds with biological activities and attractive properties for the search of novel anticancer agents. (Prachayasittikul S, 2017).

Bromodomains are highly conserved domains that are made up of approximately 110 amino acids and are composed of a left-handed bundle of four alpha helices, which are linked by ZA and BC loops that vary in sequence between the bromodomain proteins.(Zaware N, 2017; Barrero MJ, 2017; Devaiah BN, 2016; Perez-Salvia M, 2017) The bromodomain contain protein that has BET family which include BRD1, BRD2, BRD3, BRD4, BRDT among all the bet family BRD4 related cancer become the most challenging disease today because this particular bromodomain protein when it get over expressed in the human body it cause many disease such as cancer, inflammation and inflammatory related disease, Bromodomain and

Extra-terminal (BET) proteins are epigenetic readers that interact with acetylated lysines of N- terminal of histone tails.(Huijbregts L, 2019) and help in the epigenetic regulation.

Epigenetic modifications are actually reversible, heritable alterations to the DNA of a cell which don't involve a difference at the nucleotide sequence. A number of epigenetic mechanisms, which includes modifications in CpG island methylation patterns as well as histone modifications regulate gene expression and enjoy a crucial role in keeping normal cellular homeostasis. Dysregulation of proteins to blame for mingling with and modifying macro molecular complexes of DNA as well as histone proteins called chromatin is often noticed in inflammatory cells as well as cancer cells, supporting the concept that epigenetic regulation of gene expression contributes to condition called pathogenesis. (Prinjha R, 2013; Bayarsaihan D, 2011).

JQ1 is actually a bromodomain inhibitor that selectively binds to the amino acid at N-terminal of histone residue lysine, (Marazzi I, 2018) JQ1 therapy displaces BRD4, inhibiting the ability of its to examine acetylated lysine residues (Bradner JE, 2017) As a result, JQ1 selectively represses the MYC oncogene (Grivennikov SI, 2010) in an assortment of cancer cell lines as well as animal models of cancer, which includes acute myeloid leukemia (Deeney JT, 2016) likewise So other bromodomain inhibitor are intended and synthesized and therefore, are utilized for the pharmacological activity and some are actually in the clinical trial but show very less promising results due to the unclear mechanism of action and also very bad selectivity toward the target like protein, enzyme, nucleic acid, receptor, DNA etc.

We've additionally **designed 28** the pyrimidine derivative compound with help of chemdraw, (4HY3) protein was downloaded from protein data bank (PDB)(Giles D, 2015) and molecular docking were analyzed using Accelrys drug discovery studio 3.5 and based on CDOCKER Energy the most effective hit molecule was selected and synthesized for the inhibition toward bromodomain protein. The Drug likeness properties, Absorption, Distribution, Excretion and Metabolism, hepatotoxicity, plasma protein binding, aqueous solubility, blood brain barrier (BBB) carried out, It was discovered that compound 2c, 2l, 2k, 2j, 2i, 2h, 2f, 2m as well as 2s showed good CDOCKER interaction energy ranges from **29.80 to 25.06 kcal/mole**. Additionally the interaction with many amino acid for example ARG 16, SER 83 and ILE 21 whereas the standard compound JQ1 does not possess some interaction with any of the amino acid. Among them compound 2c possess good CDOCKER interaction

energy with bromodomain receptor and hence it's anticipated to bind with the receptor better compared to some other compounds. Accelrys drug discover studio 3.5. was used to calculate in-silico ADME parameters. They'd been calculated to avoid malfunction of the drug within the last phases of drug discovery process. All the designed 28 compounds possessed show good ADME properties.The aqueous solubility level 0 and 1. for 0 level value is (log(molarsolubility) <−8.0) that show extremely low whereas 1 level value is −8.0 < log(molar solubility) <−6.0) which indicate no, very low but possible and BBB level 1 and 4 the value 1 indicate high and the value 4 show undefined. Inhibition level of CYP2D6 were1 and less 1.

The 0 indicate Non-inhibitor and 1 show inhibitor. hepatotoxic level were less than 1 that show non toxic. All these recommended that the designed compounds could be druggable and hence it'd been more ready for docking studies. (Giles D, 2015).

For skin irritation test compound predicted to be not toxic in case it ranges from 0 to 0.29, between 0.3 to 0.69. The compound is actually indeterminate of course, if it ranges from 0.7 as well as 1 is actually deadly. If the discriminant score is bad then causing of cancer is actually 0 or maybe non carcinogenicity in case, the discriminant score is actually good the probability that will get cancer is actually rather high. The hydrogen bond donor ranges from 0 to 5 whereas acceptor having 3 to 8 and follow to the Lipinski rule of five as well as raises the drug likeness properties. (Giles D, 2015).

The compound **2c** was synthesized using following step, a synthetic scheme was designed and based on synthetic scheme, designed derivative compound were synthesized. These were synthesized in 2 general steps by substituted aldehyde with 1, 3 indane dione with catalytic amount of aqueous sodium hydroxide in water ethanol solvent in the ratio of 15:20 in the round bottom flask as well as even further guanidine hydroxide was added that had been refluxed for 2 h to yield **1a-1.** The compound **1a-1**. (Anbhule PV, 2014) viewed with substituted aldehyde in water ethanol solvent with few drop conc. Sulphuric acid that on reflux for 2 h to yield **2a-1**. (Shenoy S, 2009) This was recrystallized with ethanol. The improvement of the response as well as purity of the synthesized compound was administered utilizing precoated TLC plates (Funiss BS, 2004) that were visualized possibly by viewing in UV Visible or perhaps by iodine vapor chamber. Melting point (Funiss BS, 2004) of the product was measured. The distinction in the melting point as well as TLC suggests the development of completely new a compound.

Formation of pyrimidine derivative **2a-1** was confirmed by the spectra data such as IR spectra (Silverstein RM, 1991; William K, 1991) **1a-1** exhibited absorption bands at range of **3305.39 to 3485.06 cm^{-1}** as well as ^{1}H NMR spectra (Silverstein RM, 1991; William K, 1991; Sharma YR, 1998) was found to be **7.25** whereas NH wasn't observed in final product **2a-1** (Silverstein RM, 1991; William K, 1991; Sharma YR, 1998) The *in vitro* cytotoxic effect of pyrimidine derivative compound was measured by MTT assay (Scudiero D, 1988; Mosmann T, 1983; Nair L, 2011) on **five different s**elected cancer cell line such as HL-60 (Leukemia cancer cell line), HELA (Cervical cancer cell line), MCF-7 (Breast cancer cell line), HCT-116 (Colon cancer cell line), and A431 (Squamous carcinoma cell line) to determine its efficiency to inhibit the cancer cells viability in the presence of different concentrations of the inhibitor **(25 µg, 50 µg, 75 µg, 100 µg)**.The result shows that the synthesized pyrimidine derivative compound inhibits more leukemia (HL-60) cancer cell line as compared to standard 5 FU drug with increase concentration of **25 µg, 50 µg, 75 µg, 100 µg** and for the synthesized compound the highest activity is **54.99%** obtained at the concentration of **100 µg,** the lowest activity is **16.86%** obtained at the concentration of **25 µg** with **IC_{50} value 83 µMole** and for 5 FU, the highest activity is **51.1%** obtained at the concentration of **100 µg**, the lowest activity is **7.01%** obtained at the concentration of **25 µg** with the **IC_{50} value 100 µMole**.

The end result shows that the synthesized pyrimidine derivative compound inhibits much more breast cancer (MCF 7) cellular line as compared to standard **Imantinib Mesylate drug** with different concentration **(25 µg, 50 µg, 75 µg, 100 µg)**.and for the synthesized compound probably the highest activity is actually **65.9** % received at the focus of **100 µg** , probably the lowest activity is actually **43.1 %** obtained at the focus of **25 µg** with **IC_{50} value 40 µMole** and for **Imantinib Mesylate,** probably the highest activity is actually **60.2 %** obtained at the concentration of **100 µg**, probably the lowest activity is actually **7.39 %** obtained at the concentration of **25 µg** with the **IC_{50} 100 µMole** along with other **IC_{50}** value of synthesized pyrimidine for HeLa was **83 µMole** and standard 5 FU drug was **104 µMole**, **IC_{50}** value of synthesized pyrimidine compound for HCT 116 was **44 µMole** and standard Imantinib Mesylate drug was **10 µMole** and **IC_{50}** value of synthesized pyrimidine compound for A431 was **44 µMole** as well as standard Imantinib Mesylate drug was **10 µMole.**

The *in vitro* bromodomain enzyme inhibition assay was done on MCF 7 (Breast cancer cell collection) at **25µM** focus on synthesized pyrimidine derivative compound as well as typical

JQ1 inhibitor. The end result shows that at **25µM concentration**, synthesized pyrimidine derivative compound inhibits much less MCF 7 cancer cell line as in comparison to typical JQ1 inhibitor.

Acute toxicity study for synthesized pyrimidine compound had been carried out by adapting up & down technique, Committee for the purpose of Supervision and Control on Experiment on Animals (CPCSEA) institution of economic cooperation as well as development (OECD) guidelines 425. The test was done using AOT425Statpgm (Version: 1.0) and the test results were obtained based on Acute toxicity studies (OECD of guidelines 425) statistical system. The Swiss albino female mice whose body mass 20-25 g was considered for acute toxicity study. The animal were provided with all the necessary facility as per protocol of the experiment. The pyrimidine derivative compound was given orally and animals had been observed constantly for 12 h to identify some change in autonomic or maybe behavior response and go on for 24 h. The mortality rate was noticed for 48 h. as per the main test, the analysis was begun with 175 mg/kg, followed by 550 mg/kg and finally administered of 2000 mg/kg p.o with a limit serving of 2000 mg/kg. Observed the animal for 14 day for just about any toxicity sign. (Test guideline OECD 425, 2008).

The 0.2 ml of Ehrlich Ascites Carcinoma cell are actually inoculated to 24 Adult female Swiss Albino mice and divided into 3 groups, each containing 8 mice in a group. After tumor inoculation, treatments will be offered orally, as soon as every day for ten days. On the 11^{th} day, 6 animals from each group was anaesthetized and blood was collected via tail vein method. The hematological parameters including white blood cells (WBC), Red blood cells (RBC), hemoglobin (Hgb) as well as platelets (PLT) will be approximated.

The animal's life span (ALS), increase percentage life span (%ILS) as well as each group animal body weight analysis was carried out to know the effect of synthesized pyrimidine derivative compound with standard drug 5 FU (Chandran A, 2015; Islam K, 2012; Mondal A, 2016) The outcome of Hgb, RBCs and WBCs was examined utilizing EAC induced cancer cell, result shown that Hgb count was decreased in EAC cancer control mice i.e., (4.7 ± 0.30 g/dL) when compared to synthesized pyrimidine derivative compound (200 mg/kg) (10.4 ± 0.08 g/dL) and standard 5 FU (20 mg/kg) (11 ± 0.35 g/dL) on Treatment with synthesized pyrimidine derivative compound (200 mg/kg) showed an extremely significant increase (5.67 ± 0.15) $1x10^6$ $/mm^3$in RBC count as compared to EAC cancer control group (2.05 ± 0.11) $1x10^6$ $/mm^3$, while significant increase (5.99 ± 0.154) $1x10^6$ $/mm^3$ in RBC count on

administration of standard of 5 FU (20 mg/kg) the result shown that WBC count was increase in EAC cancer control mice i.e., (30.48 ± 0.45) $1x10^3/mm^3$ when compared to synthesized pyrimidine derivative compound (200 mg/kg) (17.33 ± 0.39) $1x10^3/mm^3$ and standard 5 FU (20 mg/kg) (15.63 ± 1.46) $1x10^3/mm^3$

Effect of synthesized pyrimidine compound (200 mg/kg) and standard drug 5 FU (20 mg/kg) on Mean Survival Time (MST) as well as percentage increase in life span (% ILS) in mice were induced by EAC cancer cell. (Ghai C, 1999) The end result show that in EAC cancer group mice the Mean survival time was (15 ± 0.32) days. Whereas, it had been substantially enhanced on therapy with examine synthesized pyrimidine derivative compound (200 mg/kg) by (87.5 %) (23 ± 0.57) days Whereas, on treatment with standard drug 5 FU (20 mg/kg) increase the life span by (87.5 %) and also enhanced the mean survival time considerably (22 ± 0.53) days.

The impact of synthesized pyrimidine compound (200 mg/kg) and standard 5 FU (20 mg/kg) on animal body weight was induced by EAC cancer cell (Ghai C, 1999) show that animal was induce with EAC cancer cell and divided them into 3 group, each group consist of 8 mice and the each group was named as cancer control group (0.9 % normal saline), standard group (5 FU) and test group (pyrimidine synthesized compound). The cancer mice body weight increases with regard to day increases at 0 day (35.9 ± 0.5) g, 7 days (38.9 ± 0.6) g, 14 days (40.8 ± 0.5) g as well as 21 day at 21 day. all cancer control animal were died, whereas the body weight of mice get reduction on treatment with synthesized pyrimidine derivative compound **(2C)** (200 mg/kg), zero day (30.8 ± 1.0) g, 7 days (28 ± 1.5) g, 14 days (26.8 ± 1.3) g as well as 21 days (23 ± 3.4) g as well as standard drug 5 FU (20 mg/kg), 0 day (39.1 ± 0.5) g, 7 days (37.3 ± 0.8) g, 14 days (35 ± 0.8) g and 21 days (29.1 ± 4.26) g on comparability with 0 day treatment.

The effect of synthesized pyrimidine compound (200 mg/kg) and standard drug 5 FU (20 mg/kg) was estimated for antioxidant activity against EAC model. The 24 mice were first induce with EAC cancer cell and divided into three group. the each group consist of 8 mice, the group which were divided named as cancer control group (0.9% normal saline), test group (200 mg/kg/p.o) and standard group (20 mg/kg/p.o) and the superoxide dismutase (SOD), Reduced Glutathione, Catalase, Nitric oxide activity and total protein were estimated the result found that The cancer control mice SOD level were significantly decreases in both the kidney and liver whereas the SOD level get significantly increases in both the kidney and

liver on treatment with test synthesized pyrimidine derivative compound (200 mg/kg) and standard drug 5 FU (20 mg/kg). The cancer control mice GSH level were significantly decreases in both the kidney and liver whereas the GSH level get significantly increases in both the kidney and liver on treatment with test synthesized pyrimidine derivative compound (200 mg/kg) and standard drug 5 FU (20 mg/kg).

5. CONCLUSION

From the present investigation following conclusion are drawn

- This work was designed to find out the common structure feature required to possess good anti-cancer activity.
- A Series of Novel Methoxy substituted pyrimidine derivatives compound were designed and docked on bromodomain receptor using Accelrys drug discovery studio 3.5. with PDB Code of **4HY3** for its anti-cancer activity.
- It was found that most of the designed pyrimidine derivatives compound were interacted with ARG 16, SER 83 and ILE 21 amino acids while standard JQ1 has no interaction with any of the amino acid residue thus, the designed compound **2c, 2f, 2h, 2i, 2j, 2k, 2l, 2m and 2s** showed good CDOCKER interaction energy ranges from **-29.80 to -25.06 kcal/mole** but among them compound **2c** show good CDOCKER interaction energy. Therefore, the designed compound **2c** were synthesized and characterized by physical and spectral data like Melting point, TLC, IR, ^{1}H NMR and Mass spectroscopy.
- It was observed that compound **2c** possess better anticancer activity as compared to standard 5 FU both *in vitro* and *In vivo.*
- The *in vitro* cytotoxic effect of synthesized pyrimidine derivative **(2c)** was measured by MTT assay on five different cancer cell line such as **HL-60** (Leukemia cancer cell line), **HELA** (Cervical cancer cell line), **MCF-7** (Breast cancer cell line), **HCT-116** (Colon cancer cell line), and **A 431** (Squamous carcinoma cell line) to determine its efficiency to inhibit the cancer cells. Among five different cancer cell line, the synthesized pyrimidine derivative compound **(2c)** shows good **IC_{50} value** on **HL-60** (Leukemia cancer cell line), **HELA** (Cervical cancer cell line), **MCF-7** (Breast cancer cell line) as compared to standard compound 5 FU whereas the synthesized pyrimidine derivative compound **(2c)** show not very good IC_{50} value on **HCT-116** (Colon cancer cell line), and **A 431** (Squamous carcinoma cell line) as compared to standard drug Imantinib Mesylate.
- The *in vitro* synthesized pyrimidine derivative compound **(2c)** and standard JQ1 inhibitor both were used on bromodomain enzyme inhibition assay on MCF-7 (Breast cancer cell

line) at 25µM concentration. The result shows that the synthesized pyrimidine derivative compound **(2c)** inhibits less breast cancer (MCF-7) cell line as compared to standard JQ1 inhibitor at **25 µM** concentration.

- The acute toxicity study was carried out based on (OECD guide line 425) to find the LD_{50} The synthesized pyrimidine derivative compound **(2c)** were given orally and animals were observed continuously for 12 h to detect any change in autonomic or behavior response and continue for 24 h .mortality rate was observed for 48 h. as per the main test, the study was started with 175 mg/kg, followed by 550 mg/kg and completed with administration of 2000 mg/kg p.o with a limit dose of 2000 mg/kg observed the animal for 14 day for any toxicity sign.
- The *in vivo* hematological parameter like Hbs, RBCs and WBCs were determined for its anticancer activity using EAC cell in Swiss albino mice, the Hgb count was decreased in EAC cancer control mice when compared to synthesized pyrimidine derivative compound **(2c)** (200 mg/kg) and standard 5 FU (20 mg/kg) and on treatment with the synthesized pyrimidine derivative compound **(2c)** (200 mg/kg) the RBC count are significantly increase as compared to cancer control mice. The result shown that WBCs count was increase in EAC cancer control mice when compared to synthesized pyrimidine derivative compound **(2c)** (200 mg/kg) and standard 5 FU (20 mg/kg) but on treatment it get decreased that show good anti-cancer activity.
- The effect of synthesized pyrimidine derivative compound **(2c)** (200 mg/kg) on anti-oxidant such as SOD, Catalase, Reduce glutathione, Nitric oxide and total protein against EAC cell show good activity as compared to standard compound 5FU (20 mg/kg)
- The Mean survival time result showed that in EAC cancer control mice the level was significantly decrease but on treatment with synthesized compound **(2c)** (200 mg/kg) and standard 5FU (20 mg/kg) level was increase significantly
- The effect of synthesized pyrimidine derivative compound **(2c)** (200 mg/kg) and standard 5 FU (20 mg/kg) shows increase life span and decrease the body weight which show the good indication of anti-cancer drug.

6. SUMMARY

This study mainly emphasize with an aim and objective to experimentally performed bromodomain based anti-cancer drug with better pharmacological activity and to develop the best molecule for anticancer activity and to carried out this research work both previous and new bromodomain based anticancer related review of literature and research work was

studied properly to obtained knowledge to further carried out this project work and after review all the work that was done before a novel pyrimidine derivative compound was designed based on core modification of the compound then molecular docking was performed, in this study the pyrimidine related derivative compound are designed with the help of chemsketch and protein were downloaded from protein data bank (PDB) both ligand and protein was loaded in the Accelrys discovery Studio 3.5 the best hit molecule with good CDOCKER interaction energy was considered from the series of novel pyrimidine derivative compound from series of novel pyrimidine compound, compound **2c** possess the good CDOCKER interaction energy and further the synthetic scheme was designed for synthesis of novel molecules and synthesis of compound was performed. After synthesis of pyrimidine derivative compound the characterization, purification and confirmation of molecules was done with Melting point determination, TLC with R_f values, FTIR, ^1H NMR and Mass spectra.

Acute toxicity study (OECD guideline 425) are used to fixed dose to carry out the *in vivo* study. The synthesized pyrimidine derivative compound was given orally based (OECD guideline 425) and animals had been observed constantly for 12 h to identify some change in autonomic or maybe behavior response and go on for 24 h. The mortality rate was noticed for 48 h. as per the main test, the analysis was begun with 175 mg/kg, followed by 550 mg/kg and finished with administration of 2000 mg/kg p.o with a limit serving of 2000 mg/kg. Observed the animal for 14 day for just about any toxicity sign.

The *in vitro* cytotoxic effect of synthesized pyrimidine derivative **(2c)** was assessed by MTT assay on five different cancer cell line for example **HL 60 (Leukemia cancer cell), HELA (Cervical cancer cell line), MCF 7 (Breast cancer cell line), HCT 116 (Colon cancer cell line), along with a 431 (Squamous carcinoma cellular line)** to figure out the efficiency of its to prevent the cancer cells. Among five different cancer cell, the synthesized pyrimidine derivative compound (2c) shows better IC_{50} value on HL 60 (Leukemia cancer cell collection), HELA (Cervical cancer cell line), MCF 7 (Breast cancer cell line) as in comparison to standard drug 5FU whereas the synthesized pyrimidine derivative compound (2c) show less IC_{50} on HCT 116 (Colon cancer cell line), along with a 431 (Squamous carcinoma cell line) as in comparison to standard drug Imantinib Mesylate.

The *in vitro* enzyme inhibition assay on MCF 7 (Breast cancer cell line) was carried out at 25 µM for both synthesized compound (2C) and standard JQ1 inhibitor and it was observed that

synthesized compound (2C) show less inhibition as compared to standard JQ1 inhibitor and *in vivo* activity was carried out with compound (2C) (200 mg/kg) and standard drug 5 FU (20 mg/kg) on hematological parameter like Hbs, RBCs, WBCs and the result suggested that Hbs and RBCs count was less in cancer control mice but on treatment with synthesized compound (2C) (200 mg/kg) and standard drug 5 FU (20 mg/kg) their count was increase whereas in case of WBCs initially their white blood cell count was more in cancer control group but on treatment with synthesized compound (2C) (200 mg/kg) and standard drug 5 FU (20 mg/kg) their count decrease that show drug is showing good activity. the animal body weight were increase in cancer control mice as the day increase whereas on treatment with both synthesized compound **(2C)** (200 mg/kg) and standard drug 5 FU (20 mg/kg) the animal body weight get decrease with normal body weight as the day increases that show good effect of drug on animals. Similarly for antioxidant activity such as superoxide dismutase, Reduce glutathione, Catalase, Nitric oxide and Total protein with EAC induce mice show excellent anticancer activity as compared to standard compound 5FU (20 mg/kg) because the superoxide dismutase, Reduce glutathione, Catalase, Nitric oxide are less in cancer control mice but on treatment with both synthesized compound **(2C)** (200 mg/kg) and standard drug 5 FU (20 mg/kg) it get increased. Whereas, total protein was more in cancer control mice but on treatment both synthesized compound **(2C)** (200 mg/kg) and standard drug 5 FU (20 mg/kg), it get drastically decrease that show the synthesized compound has good anti-cancer activity as compared with standard compound 5 FU.

Future Research

More experimental work such as Western blot analysis, Real-time PCR, Flow cytometer, ELISA analysis, histologic analysis and Immunofluorescence, Chromatin Immuno precipitation and Immuno cytochemistry is needed to carry out to find the more pharmacological feature required for the drug to possess good anti-cancer activity.

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