BIOLOGICAL EVALUATION OF LIBRARY OF NATURAL AND SYNTHETIC COMPOUNDS

A dissertation
In partial fulfilment of the requirement
For the award of the degree of
Masters of Science in Biotechnology

Under the guidance of
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CERTIFICATE

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It is certified that the work contained in the thesis entitled "**Biological evaluation of Natural and Synthetic compounds**", by '**Nikita**', has been carried out under my/our supervision and that this work has not been submitted elsewhere for a degree.

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This is to certify that the matter in the thesis entitled "Biological evaluation of Natural and Synthetic Compounds" has been carried out by Nikita (Roll no. 301201012), under our guidance and supervision for the partial fulfilment of Master of Sciences in Biotechnology, Thapar University (Deemed University), Patiala.

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DECLARATION

I hereby declare that the work which is being presented in this thesis **" Biological Evaluation of Natural and Synthetic compounds"** submitted by the undersigned in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. Avinash Bajaj,** Assistant Professor, Regional Centre for Biotechnology, Gurgaon, Haryana, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

Date: July 18, 2014

Nikita

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Table of Contents

- Conclusion
- Refrences

List of figures

List of Tables

CHAPTER 1
INTRODUCTION

1.1 CANCER

Cancer[1], the most widely studied disease of the present millennium, is known to cause due to a series of genetic alterations, causing a loss of normal growth controls, resulting in unregulated growth, lack of differentiation, apoptosis, genomic instability and metastasis. Cancer can grow in any tissue of the body at any age. But the most differentiated and unique property of the cancerous cells is that they have a long latent period with no obvious traces of the disease. Epidemiological studies have proved that chances of cancer increases with increase in age. At the molecular level, in order to gain the proliferative advantage required for tumorigenesis mutations should be caused in genes responsible for cell cycle progression and growth. The two major categories of genes altered in cancer are the oncogenes and tumor suppressor genes.

- **Tumor suppressor genes:** These are genes which hinder cell division and survival.

- **Proto-oncogenes:** These are genes which encode proteins that stimulate cell growth and reproduction

Cancer types can be grouped into broader categories. The main categories of cancer include:

- **Carcinoma** – cancer that begins in the skin or in tissues that line or cover internal organs. There are number of subtypes of carcinoma, including adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma.

- **Sarcoma** - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.

- **Leukemia** - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.

- **Lymphoma and myeloma** - cancers that begin in the cells of the immune system.

- **Central nervous system cancers** - cancers that begin in the tissues of the brain and spinal cord.

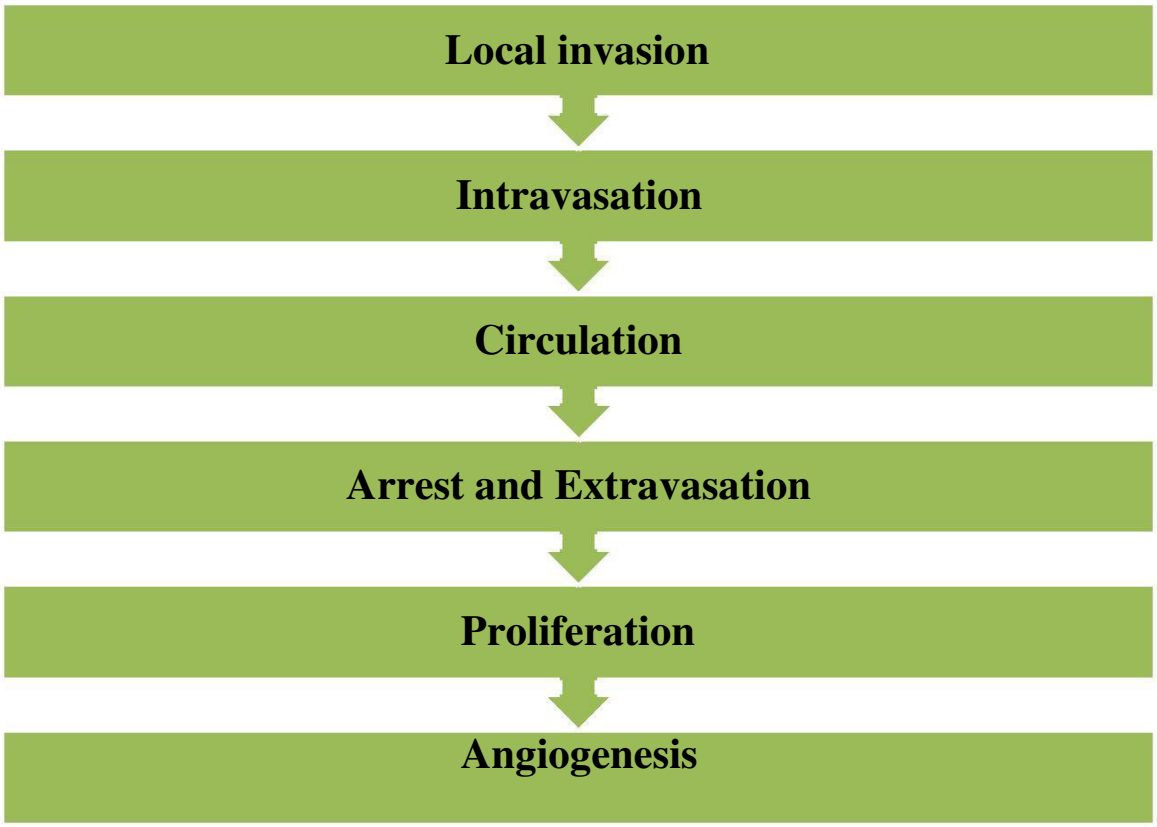
There are three basic characteristics of cancerous cells:-

Angiogenesis- tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste

products. Tumor angiogenesis actually starts with cancerous tumor cells releasing molecules that send signals to surrounding normal host tissue. This signaling activates certain genes in the host tissue that, in turn, make proteins to encourage growth of new blood vessels. The walls of blood vessels are formed by vascular endothelial cells. These cells rarely divide, doing so only about once every 3 years on average. However, when situation requires it, angiogenesis can stimulate them to divide. Angiogenesis is regulated by both activators and inhibitors. Inhibitors help in stopping the growth of vessels and activators help in producing new blood vessels and there is a decrease in inhibitors. Some of the activators of angiogenesis are Vascular Endothelial Growth Factor (VEGF), basic Fibroblast Growth Factor (bFGF) etc. Some of the inhibitors are angiostatin, endostatin, platelet factor IV etc.

Metastasis [2]**:** It's a process in which cancer cells spread from its original location to some other location in the body. A tumor formed by metastatic cancer cells is called a metastatic tumor and the process of invasion of cells to other part of the body to cause cancer is called metastasis. There are many types of cancers that can be metastatic; mainly breast, colon, kidney and lungs.

Cancer cell metastatis usually involves the following steps:



Suppression of Apoptosis [3, 4]**:** Apoptosis is the self-destruction program for safely removing the defective or damaged cells before they can spread the abnormality by dividing and replicating. These cells can be safely discarded without incurring any collateral damage to healthy neighbouring cells. This system is a very effective defense mechanism against cancer, since genetic defects occur early in the cancerous process. Apoptosis is the natural or healthy end of a malignant or pre-malignant cancer cell. For cancer to become dangerous, it must develop ways to override the program that leads to apoptosis. Interfering with the apoptotic pathway itself is the most direct approach cancer uses to override this protective process. The main two protein families responsible for the process of apoptosis are the pro and anti-apoptotic proteins. A disturbance in balance between these two can lead a cell towards cancer.

Therapeutic strategies of cancer treatment:

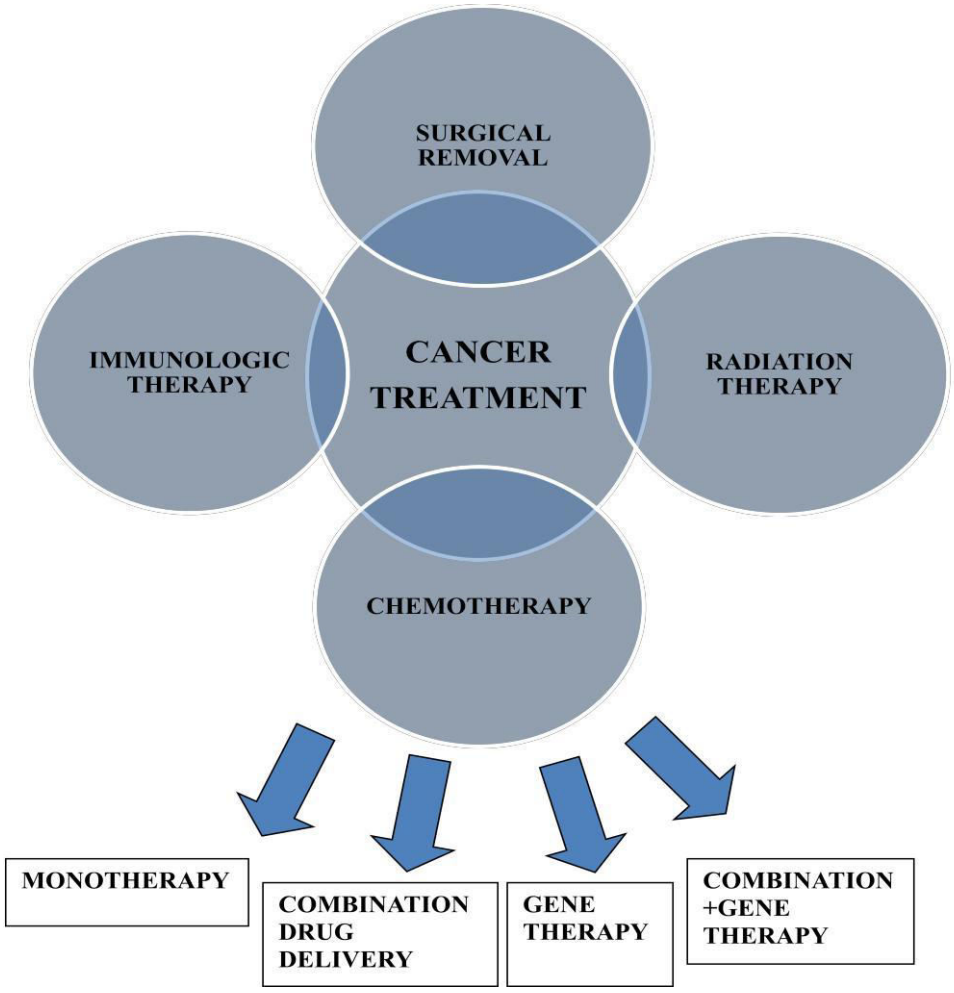


Figure 1.1: Therapeutic Strategies in Cancer Therapy[5]

- **Surgery**[6]**:** it is the most common treatment for all stages of colon cancer. Following types of surgery can be used:
  - Local excision: this type of surgery is being done in the early stage of colon cancer, where cut in the abdominal wall is being avoided.
  - Resection of the colon with anastomosis: defected area or tumor part of the colon is being dissected and healthy parts are sewed (anastomosis).
- Radiofrequency ablation- use of special probe with tiny electrodes that kill cancer cells.
- Cryosurgery- it is a treatment that uses an instrument to freeze and destroy abnormal tissue.
- Chemotherapy- this is the most common method used for the treatment of cancer and it does not include any kind of cuts or bruises in the patient's body. There are number of drugs which are being used for the treatment and they either stop the cell division or kill the cells in effected area. Drugs enter through bloodstream and reach the cancer cells throughout the body.
- Radiation therapy- it uses high-energy x-rays or other types of radiation to kill cancer cells or keep them from growing.
- Targeted therapy- it uses drugs or other substances to identify and attack specific cancer cells without harming normal cells. It includes; monoclonal antibodies, angiogenesis inhibitors.

1.1.1 COLON CANCER

Cancers of colon and rectum are the second leading cause of deaths in the world with a 6% lifetime risk of developing disease. Tumor suppressor genes, proto oncogenes, DNA repair genes, growth factors and their receptor genes, cell cycle checkpoint genes, and apoptosis related genes are mutated genes at different stages of colorectal cancer development[6]. Most colorectal cancers arise from adenomatous polyps-clusters of abnormal cells in the glands covering the inner wall of the colon. There is a massive decline in the death rate from colorectal cancer in the last decades which speak to the potential effectiveness of recent advances in prevention, screening, and therapy. Cancers of the colon arise from the colonic epithelial cells that line the lumen of the organ which renew themselves every five days from a stem cell population located at the base of colonic epithelial cell crypts.

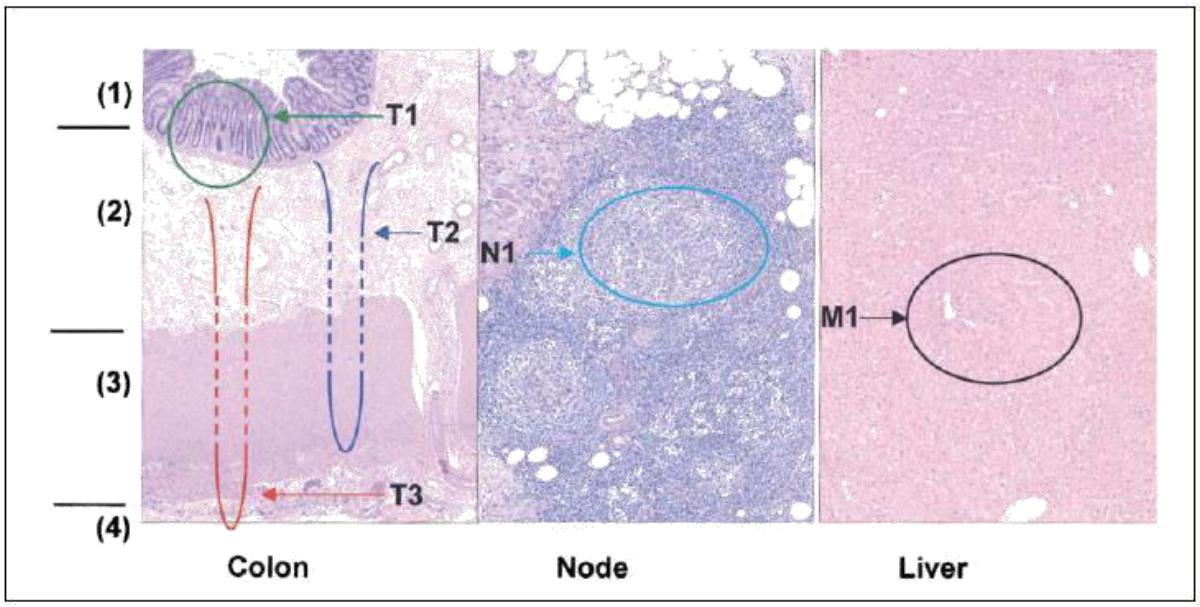


Figure 1.2: Clinical staging of colon relative to the histology of the normal colon. Shown are the principle layers of the colon including the mucosa (1), the submucosa (2), the muscle of the muscularis propria (3), and the subserosa (4). Also shown are micrographs of a lymph node in the coloric mesentry, and of the distant liver. The outlines show the extent of spread of different potential tumors and their stages as defined by the commonly employed TNM (tumor, nodes, and metastasis) staging system[7].

Stages of Colon Cancer

Staging is the process of determining how far a tumor has spread beyond its original location. Staging may not be related to the size of the tumor. Treatment decisions also depend upon the stage of a tumor. Staging for colon cancer is as follows:

- Stage 0- the cancer is found only in the innermost lining of the rectum or colon.
- Stage 1- cancer has spread from the mucosa of colon wall to muscle layer.
- Stage 2- the cancer has spread through muscle layer of colon to serosa
- Stage 3- the cancer has spread to submucosa and one lymph node atleast.
- Stage 4- the cancer has spread through the blood and lymph nodes to other parts of the body. This stage is not dependent on how far the tumor has penetrated or if the cancer has spread to lymph nodes near the tumor.

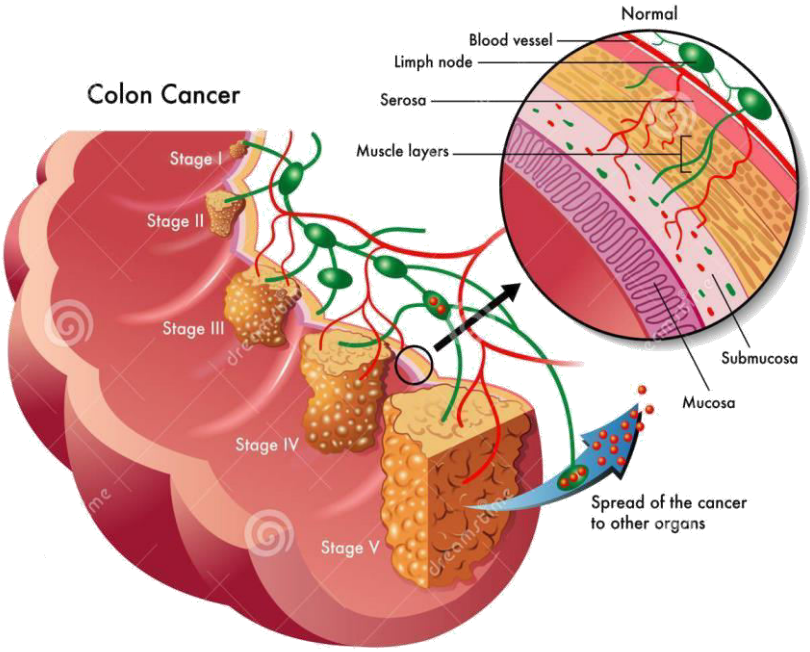


Figure 1.3: Development of stages of colon cancer

Symptoms

- Diarrhoea or constipation
- A feeling that your bowel does not empty completely
- Blood (either bright red or very dark) in your stool
- Frequent gas pains or cramps, or feeling full or bloated
- Weight loss with no known reason
- Fatigue
- Nausea or vomiting

1.1.2 ANTICANCER AGENTS

Anticancer agents or anticancer drugs are the one which are used to develop the therapy for cancer treatment. There are various compounds which can be isolated from natural resources and then can be modified to develop an anticancer drug. The discovery and development of anticancer drugs, especially cytotoxic agents, differ significantly from the drug development process for any other indication. The available anticancer drugs have distinct mechanisms of action which may vary in their effects on different types of normal and cancer cells. A single "cure" for cancer has proved elusive since there is not a single type of cancer but as many as 100 different types of cancer. In addition, there are very few demonstrable biochemical

differences between cancerous cells and normal cells. For this reason the effectiveness of many anticancer drugs is limited by their toxicity to normal rapidly growing cells in the intestinal and bone marrow areas. A final problem is that cancerous cells which are initially suppressed by a specific drug may develop a resistance to that drug. For this reason cancer chemotherapy may consist of using several drugs in combination for varying lengths of time. For example: Imatinib nesylate, Gefitinib, Bortezomib, Rituximab etc.

The role of anticancer drugs is to slow and hopefully halt the growth and spread of a cancer. There are three goals associated with the use of the most commonly-used anticancer agents.

- Damage the DNA of the affected cancer cells.
- Inhibit the synthesis of new DNA strands to stop the cell from replicating, because the replication of the cell is what allows the tumor to grow.
- Stop mitosis or the actual splitting of the original cell into two new cells. Stopping mitosis stops cell division (replication) of the cancer and may ultimately halt the progression of the cancer.

Natural compounds in cancer therapy

The role of natural products as a source for remedies has been recognized since ancient times [8]. There has been a major scientific and technological progress in combinatorial chemistry; drugs that are being derived from natural sources are making the major part of drug discovery today. Tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms which serve as a strong therapeutic means. This chemical diversity is present in natural sources such as plants to repel the predators, but discoveries and research led to the use of this mechanism for disease treatment. Combinatorial synthesis, high-throughput screening, and their associated approaches are the new technologies which lead to the development of natural-product-based drug discovery. Vincristine, irinotecan, etoposide and paclitaxel are classic examples of plant-derived compounds; actinomycin D, mitomycin C, bleomycin, doxorubicin and l-asparaginase are drugs coming from microbial sources, and citarabine is the first drug originating from a marine source. To date, new generations of taxanes, anthracyclines, Vinca alkaloids, camptothecins, as well as the novel class of epothilones have been developed. Some of these are in clinical use, others in clinical trials [9].

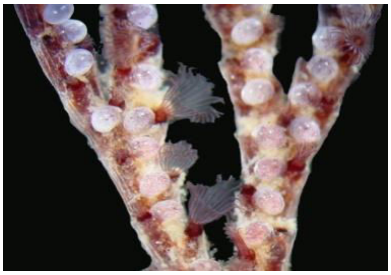
Cataranthus roseus

Podophyllum peltatum

(A)

Taxus brevifolia

Camptotheca acuminata



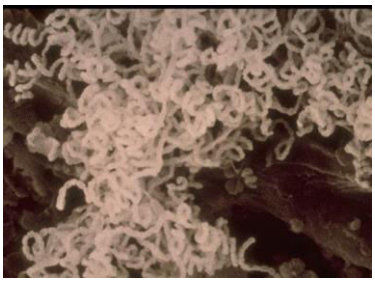
Bugula neritina

Aplidium albicans

(B)

Dolabella auricularia

Ecteinascidia turbinata



Actinomyces antibioticus

(C)

Figure 1.4: Main examples of plant (A) microbial (B) and marine (C) sources of anticancer agents[9]

During the last decade, works on natural compounds have been particularly successful in the field of anticancer drug research. Camptothecin is an example of naturally driven anticancer drug; it is a monoterpenoid alkaloid, isolated from the Chinese ornamental tree *Camptotheca acuminata* in the sixties. Two derivatives irrinotecan (a pro-drug) and topotecan were developed from camptothecin and they were less toxic and more stable than camptothecin itself. They are currently used as anticancer agents, for the treatment of colorectal and ovarian cancers in particular. Taxols (paclitaxel and the semi-synthetic derivative docetaxel), *Vinca* alkaloids (vinblastine, vincristine, and the semi-synthetic derivative vinorelbine), podophyllotoxins (the semi-synthetic derivatives: etoposide and teniposide), are also among the most frequently used anticancer drugs [10].

1.2 MICROBIAL INFECTIONS

There are different types of microorganisms present in the environment that causes a large number of diseases. From common cold to tuberculosis, from normal to deadly diseases, microbes are responsible. Microbes can be divided into four broad classes:

- Bacteria
- Viruses
- Fungi
- Protozoa

Bacteria are unicellular organisms. They possess naked DNA. They are larger in size than viruses. Bacteria produce **spores**, and these spores are inactive but can tolerate chemicals as well as high temperature.

Not all bacteria are disease causing microbes; some of them are useful for the human beings. Gut microflora contains bacteria which work for the welfare of human beings. The bacteria which infect the body, produces toxins which affect the body and these toxins can be killed by antibiotics. Bacteria can attack any part of the body. Maximum diseases are being caused by the bacteria only.

Examples of *diseases* caused by bacteria: cholera, tuberculosis (TB), septicaemia ("blood poisoning"), anthrax.

Viruses are very small in size and cannot be seen through naked eyes. They are obligate parasites. They actively reproduce inside the host body but remain inactive in outside environment. Some of the deadly diseases are being caused by viruses only.

Examples of *diseases* caused by viruses**:** flu (influenza), common cold, measles, mumps, german measles (Rubella), smallpox, cowpox, chicken pox, HIV (can lead to AIDS), rabies

Fungi are slightly larger than bacteria, and their DNA is contained inside nuclei like plant and animal cells. Fungi consist of hypha which penetrates through the body and infects the cells.

Examples of *diseases* caused by fungi: Athlete's foot, ringworm, thrush

Protozoa/protoctista resembles to our body cells because they consist of single cell only with true nuclei and a cell membrane.

Examples of *diseases* caused by protozoa: Malaria, sleeping sickness, dysentery

1.2.1 ANTIMICROBIAL AGENTS

Antibacterial therapy, a keystone in modern medical practice, provides one of the pharmacological treatments that cure bacterial diseases. Antimicrobial agents are those compounds which interfere with the growth and reproduction of microorganisms. [11]Most microbiologists distinguish two groups of antimicrobial agents used in the treatment of infectious disease: **antibiotics**, which are natural substances produced by certain groups of microorganisms, and **chemotherapeutic agents**, which are chemically synthesized. A hybrid substance is a **semisynthetic antibiotic**, wherein a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties. The modern era of antimicrobial chemotherapy began in 1929, with Fleming's discovery of the powerful bactericidal substance, penicillin, and Domagk's discovery in 1935 of synthetic chemicals (sulfonamides) with broad antimicrobial activity.

Although there are a number of different types of antibiotic, they all work in one of two ways:

- A **bactericidal** antibiotic kills the bacteria. A bactericidal usually either interferes with the formation of the bacterium's cell wall or its cell contents. This makes a

bacterium membrane compromised and cell death occurs. For example; penicillin, vancomycin, daptomycin etc.

- A **bacteriostatic** stops bacteria from multiplying and reproducing and hence decreasing the population number of the bacteria. On removal of bacteriostatic antibiotic, bacteria start growing again. These antibiotics interfere with the protein production, DNA replication, or other aspects of bacterial cellular metabolism and inhibit its growth. For example; tetracycline, sulfonamides, chloramphenicol etc.

Mechanisms of Action of Antimicrobial Agents

Antimicrobial agents should act selectively on vital microbial functions with minimal effects or without affecting host functions. Different antimicrobial agents act in different ways. However, the mechanism of action of antimicrobial agents can be categorized further based on the structure of the bacteria or the function that is affected by the agents. These include generally the following:

- Inhibition of the cell wall synthesis
- Inhibition of ribosome function
- Inhibition of nucleic acid synthesis
- Inhibition of folate metabolism
- Inhibition of cell membrane function

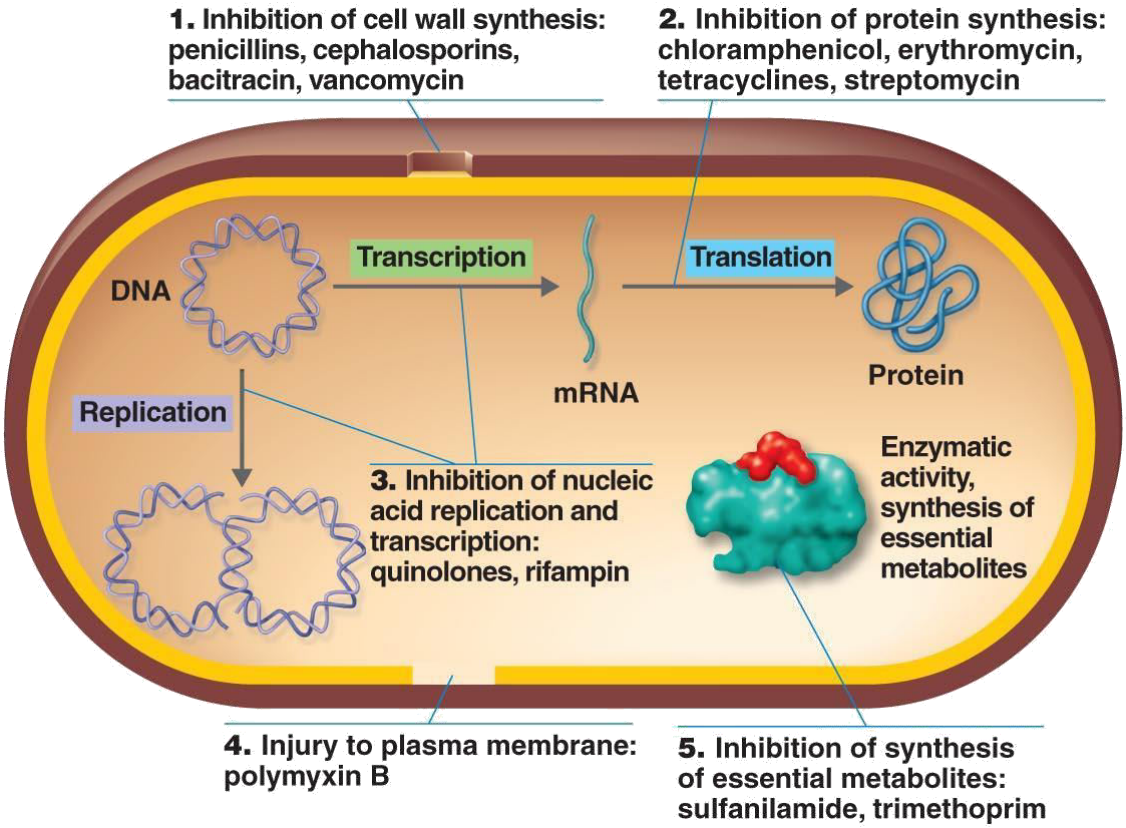


Figure 1.5: Action of antimicrobial agents[12]

Features of antimicrobial drugs/agents

- **Selective toxicity:** Drug kills pathogens without damaging the host.
- **Therapeutic index:** ratio between toxic dose and therapeutic dose or ratio of LD50 to ED50. High therapeutic index → less toxic.
- **Antimicrobial action –** Bacteriostatic vs. bactericidal
- **Activity Spectrum –** Broad-spectrum vs. narrow-spectrum
- **Tissue distribution, metabolism, and excretion –** Unstable in acid; half-life duration

1.3 NATURAL COMPOUNDS

Natural compounds are those which are being produced by plants and animals. Environment has a majority of natural sources from which products can be isolated. From centuries, people are using these natural products for different purposes such as in medicine. Wide variety of

plants including algae and weed serve as a source for extracting novel natural products for various uses in human welfare.

Natural compounds have served as a major resource of drugs for human beings. Drugs which are made by isolating the natural products from plants have formed the basis of traditional medicine systems[13]. Today plant-based drugs continue to play an essential role in health care. World Health Organization has estimated that 80% of the population of the world rely mainly on traditional medicines for their primary health care.[14] Currently at least 119 chemicals, derived from 90 plant species, can be considered as important drugs in one or more countries.[15]

More than 250,000 species of higher plants are present in the world, and almost every plant species has a unique collection of secondary constituents distributed throughout its tissues. A proportion of these metabolites are likely to respond positively to an appropriate bioassay, however only a small percentage of them have been investigated for their potential value as drugs. Still, much of the marine and microbial world is still unexplored, and there must be plenty of bioactive compounds such as secondary metabolites are awaiting discovery. Many of the natural products can also serve as pharmacophores for the design and synthesis of novel substances for medical uses.

Naturally derived compounds have played an important role in the development of over 60% of clinically useful anti-cancer agents, while a considerable number of natural products or analogs derived there from are in clinical and preclinical development. Nature is being the most important resource for human beings to rely upon, for basic needs and not least, medicines.

Drugs developed from natural sources

DRUG	MEDICAL USE	MECHANISM OF ACTION	SOURCE
Aspirin	Analgesic, anti-inflammatory, antipyretic	Inhibition of COX	Plant
Atropine	Pupil dilator	Antagonist of Ach at muscarinic receptors at post-ganglionic parasympathetic neuro effector sites	Plant
Caffeine	Stimulant	Adenosine receptor antagonist	Plant
Codein	Analgesic, antitussive	Opoid receptor agonist	Plant
Digoxin	For atrial fibrillation and CHF	Inhibition of the Na+/K+ ATPase membrane pump	Plant
Pilocarpine	Glaucoma	Muscarinic receptor against	Plant
Quinine	Malaria prophylaxis	Inhibition of protein synthesis in the malaria parasite	Plant
Taxol	Anticancer agent	Antimitotic agent	Plant
Tetracycline	Antibiotic	Inhibition of protein synthesis by binding to the ribososme 30S	Microbe

		subunit	
Eugenol	Toothache	Reduces excitability of sensory nerves	Plants
Penicillin	Antibiotic	Inhibition of synthesis of cell wall peptidoglycan	Microbe

Table 1.1: Drugs and their medical use (developed from natural sources) [16]

1.4 SYNTHETIC COMPOUNDS

Synthetic compounds are referred to those compounds which are made by making alterations in chemical structures of naturally isolated compounds. Synthetic or semi-synthetic compounds are formed by altering the structures of naturally isolated compounds. Natural compounds are being isolated from plants and microorganisms serve as pharmacophores to synthesize new molecules for drug development. Most of the drugs which are being used nowadays are synthetically modified chemical structures of natural products. They are quite specific in their action. The main advantage of using synthetic compounds to make drugs is the target specificity. The basic structural moiety is always taken from the naturally isolated compounds, and with some minor or major changes they are being converted into synthetic compounds which serve as a potent anticancer or antibacterial agent.

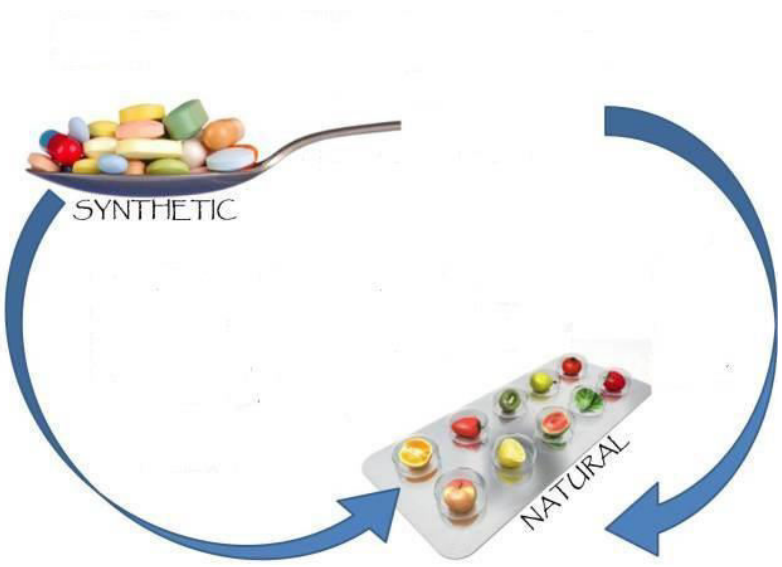


Figure 1.6: Natural to Synthetic drugs

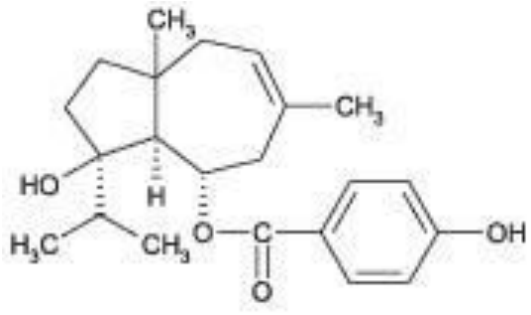
CHAPTER 2
REVIEW OF LITERATURE

➢ **Investigating anticancer properties of the sesquiterpene Ferutinin on colon carcinoma cells, *in vitro* and *in vivo*; Nahid Arghiania, Maryam M. Matin, Ahmad Reza Bahrami, Mehrdad Iranshahi, Ameneh Sazgarnia, Fatemeh B. Rassouli, *Life Sciences,***

20 June, 2014

Aim: In this research, Ferutinin was evaluated for its possible cytotoxic and apoptotic inducing effects in vitro and in vivo.

Ferutinin is a potent, naturally occuring non-steroid estrogenic compound. It is agonist for estrogen receptor (ER) α and agonist/antagonist for ERβ with higher binding affinity than tamoxifen for both ERs.



Ferutinin

This group has used Ferutinin to look for anticancer properties in vivo and in vitro. They have isolated Ferutinin from the roots of *Ferula ovina*. The genus *Ferula* belongs to the family Umbelliferae. They are rich in terpenoids such as sesquiterpene derivatives. Sesquiterpenes from *Ferula* species have antileishmanial and cancer chemopreventive activities. These compounds can induce apoptosis. Induction of apoptosis in cancer cells can increase efficiency of cancer chemotherapy. Ferutinin is a natural sesquiterpene with antimycobacterial antifungal, anti-inflammatory and apoptosis inducing activities. This sesquiterpene has shown cytotoxic activities on human and mouse leukaemia cell lines. Furthermore, ferutinin exhibits antiproliferative effects on MCF-7 cells. This group has performed MTT assay on CT 26 and HT 29 colon cancer cell lines of human and mouse o know the possible cytotoxic effects of Ferutinin. Also, DAPI staining and propidium iodide (PI) staining in vitro were performed on CT 26 cells.

After all these experiments they have concluded that there is a need for development of new anticancer drugs from medicinal plants. Sesquiterpenes, which are formed from the

assembly of three isoprene units, can be produced by plants. Ferutinin, therefore, showed selective toxicity against cancer cells.

- **Activation of JNK Contributes to Evodiamine-Induced Apoptosis and G2/M Arrest in Human Colorectal Carcinoma Cells: A Structure-Activity Study of Evodiamine; Chih-Chiang Chien *et al* , *PLOS one*, June 2014**

Evodiamine (EVO) is a natural chemical isolated from *Evodia rutaecarpa*, and several biological effects of EVO including antitumor, antinociceptive, and vasorelaxant properties were reported. This group has studied the anticancer effect of EVO on COLO 205 and HT 29 colon cancer cell lines through MTT assay and lactate dehydrogenase release assay. They have seen that the viability of colon cancer cells was inhibited by this natural molecule. They have also performed western blotting and cell cycle analysis assay through flow cytometry with same molecule on same cell lines. Induction of extracellular signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) protein phosphorylation was detected in EVO-treated cells. Data of the structure-activity analysis showed that EVO-related chemicals containing an alkyl group at position 14 were able to induce apoptosis.

On final remarks, they have concluded that in present study, EVO possesses antitumor activities including apoptosis and G2/M arrest against the viability of colorectal carcinoma cells. They have also used the natural as well as synthetic compounds for the evaluation of anticancer activity on colon cancer cell lines. They chemically modified the EVO structure to make some synthetic compounds such as alkyl group at position 14, which showed better apoptotic activity.

- **Synthesis and antitumor activities of novel a-aminophosphonate derivatives containing an alizarin moiety; Man-Yi Ye *et al, European Journal of Medicinal Chemistry*, June 2014**

This group has designed a series of novel α- aminophosphonate derivatives which have an alizarin moiety. These compounds were synthesized to check the anticancer property. Natural products are a good traditional source of new antitumor agents. Alizarin is a kind of anthraquinone which exists in rhubarb and aloe plant. Anthraquinones are widely used in the pharmaceutical industry. This group developed some derivatives to check biological activities such as antimicrobial, anti-leukemia, antioxidant, anti-HIV and anticancer activities. They have developed four different compounds and evaluated them by MTT assay against KB, NCI-H460, HepG 2, A549, MGC-803, Hct-116, CNE and Hela tumor cell lines and HUVEC

normal cells line, with 5-fluorouracil (5-FU) as the positive control. These compounds showed high inhibitory activity against these cell lines as compared to alizarin alone. Afterwards they have performed several experiments such as fluorescent staining experiments; Hoechst 33258 staining, mitochondrial membrane potential staining to check the cytotoxicity of these compounds. Apoptosis analysis and cell cycle analysis experiments were also performed through FACs.

On final remark, they have concluded that most of the derivatives showed high cell viability inhibition then the control drug 5-FU. Some compounds were analyzed for apoptotic activity through fluorescent staining, and they too showed high activity.

- **Antitumor Activity of Ethanolic Extract of *Dendrobium formosum* in T-Cell Lymphoma: An *In Vitro* and *In Vivo* Study, Ritika Prasad and Biplob Koch, *BioMed Research International*, May 2014**

Dendrobium, a genus of orchid, was found to possess useful therapeutic activities like anticancer, hypoglycaemic, antimicrobial, immunomodulatory, hepatoprotective, antioxidant, and neuroprotective activities. The study done by this group was aimed to evaluate the anticancer property of the ethanolic extract of *Dendrobium formosum* on Dalton's lymphoma.

In vitro cytotoxicity was determined by MTT assay, apoptosis was determined by fluorescence microscopy, and cell cycle progression was analysed using flow cytometry; *in vivo* antitumor activity was performed in Dalton's lymphoma bearing mice. The IC_{50} value of ethanolic extract was obtained at 350 g/mL in Dalton's lymphoma cells. Fluorescence microscopy analysis showed significant increase in apoptotic cell death in dose- and time-dependent manner which was further confirmed through the resulting DNA fragmentation. Further, flow cytometry analysis showed that the ethanolic extract arrests the cells in G2/M phase of the cell cycle. The *in vivo* anticancer activity study illustrates significant increase in the survival time of Dalton's lymphoma bearing mice on treatment with ethanolic extract when compared to control. These results substantiate the antitumor properties of ethanolic extract of *Dendrobium formosum* and suggest an alternative in treatment of cancer. Further studies are required regarding the isolation and characterization of bioactive components along with the analysis of molecular mechanism involved. Through this part of literature, one can conclude that abstracts from natural sources can be used as an effective therapeutics against many diseases and mainly against cancer. Also, this group has further evaluated some synthetic compounds evolved from same source by modifying the chemical structures. Both *in vivo* and *in vitro* studies were performed.

CHAPTER 3
MATERIALS AND METHODS

3.1 ANTIBACTERIAL STUDIES

3.1.1 MATERIALS

(A) GROWTH MEDIUM

The most commonly used growth media for microorganisms are nutrient broths and nutrient agar. Luria broth (liquid nutrient media) is widely used for this purpose. They are mostly mixed with agar and poured into Petri dishes to prepare agar plates for bacterial culture work. They remain solid, as very few bacteria are able to decompose agar. Bacteria grew in liquid cultures often form colloidal suspensions. [18]

❖ **LYSOGENY BROTH/ LURIA BROTH**

Composition of LB (1 litre):

- 10 g tryptone
- 5 g yeast extract

} Mixed in 1 litre distilled water

- 10 g NaCl
- Autoclaved at 121 °C for 20 mins
- After cooling, the LB was ready for use

LB medium is a rich medium that is commonly used to culture members of the *Enterobacteriaceae*. It is used to culture a variety of facultative organisms.

❖ **LURIA AGAR**

Yeast extract is present in Luria Agar, which makes it nutritionally rich. *E. coli* strains grow more rapidly in this medium because all the nutrients are already present in it and they don't need to synthesise any of it including Vitamin B. Sodium chloride is also present which maintains the osmotic equilibrium.

Composition of Luria Agar (1 litre)

- 10 g tryptone
- 5 g yeast extract
- 10 g NaCl
- 15 g Agar

} Mixed in 1 litre distilled water

- Autoclaved at 121 °C for 20 mins.

- Poured in Petri dishes about 25 ml in each plate.

❖ MUELLER-HINTON BROTH

Mueller-Hinton agar is a microbiological growth medium that is commonly used for antibiotic susceptibility testing.

Composition of MH broth (1 litre):

- 300 gm beef infusion
- 17.50 gm casein acid hydrolysate
- 1.50 gm starch
- Autoclaved at 121 °C for 20 mins.

(B) OTHER MATERIALS

❖ Reagents and Chemicals

$BaCl_2$, H_2SO_4, DMSO (Dimethyl sulfoxide), Penicillin, Ciproflaxin.

❖ Glassware and Apparatus

Petri plates (90 mm), Multichannel pipette, 25 Falcon tubes (50 ml and 15 ml), eppendorfs, conical flasks, vortex

❖ Bacterial Cultures

Bacterial cultures *E. coli* (MTCC 443) and *S. aureus* (MTCC 737) (procured from IMTech, Chandigarh) were used for antimicrobial susceptibility testing.

The various methods used for the antibacterial screening in this project are:

3.1.2 METHODS

(A) CFU COUNT

- **Preparation of media**: Luria broth and Luria agar media were prepared for this experiment by dissolving commercial dry media in 1 l of distilled water. Both types of media were then autoclaved.
- **Preparation of plates**: 90 mm petri plates were taken and autoclaved Luria agar media was poured into plates. About 25 ml of media was poured in each plate.

- **Preparation of inoculum**: Strain 443 and 737 were inoculated in fresh LB media. 100 µl of bacterial strain in 5 ml of fresh LB media. Both the cultures were grown overnight and then serially diluted upto 10^7 dilutions.

- **Spreading of culture**: 100 µl of each dilution was taken and spread uniformly on different LA plates. Incubated overnight at 37^o C.

- **Analysis and cfu count**: by making a quadrant on each plate, bacterial colonies were counted and result was noted.

(B) MICROBROTH DILUTION METHOD

In this test 96- well plate is used. It includes the susceptibility testing of different antibiotics on the same plate. Two-fold dilution of antibiotic is prepared and tested against test organisms. Only a small amount of antibiotic is sufficient for the test. MHB is used in this method. This test is quite sensitive and helps in the determination of MIC. The concentration at which inhibits bacterial growth or turbidity is less, that concentration is considered to be minimum inhibitory concentration.

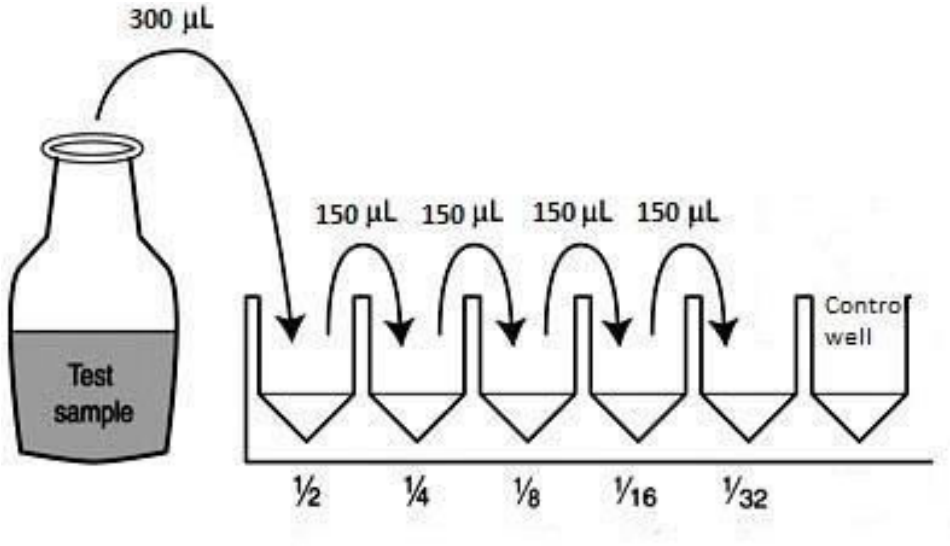


Figure 3.1: Microbroth dilution in 96- well plate

- **Preparation of media**: For this experiment Mueller Hinton broth media was prepared by dissolving the powdered media in distilled water and autoclaving it at 121^o C for 15 min.

- **Preparation of inoculum**: McFarland standard was prepared and OD was taken along with both the bacterial strains 443 and 737. 1 x 10^5 cells/ml are required for the experiment; therefore, the cultural strains were diluted accordingly to get the required number of cells.

- **Preparation of antibiotic stocks**: Stocks of Ampicillin and Kanamycin were prepared to work as controls. Stocks of test molecules were also prepared in DMSO.

- **Test protocol**:

  - All above preparations were done in laminar air flow bench, including the rest of the protocol.
  - 200 µl of MH broth was added to 12^{th} row of a 96- well plate.
  - 150 µl of MH broth was added to each well except 6^{th} and 11^{th} row.
  - Each plate was used to test 4 compounds at once.
  - In 5^{th} and 11^{th} row, 300 µl of test compounds were added in a quadrant manner.
  - Using a multichannel pipette the test compounds were serially diluted in the plate.
  - In the end, 150 µl of bacterial culture was added to each well except 12^{th} row.
  - Plates were incubated overnight at 37^{o} C on a shaker incubator.

- **Data analysis**: Next day the plates were read on spectrophotometer at 600 nm.

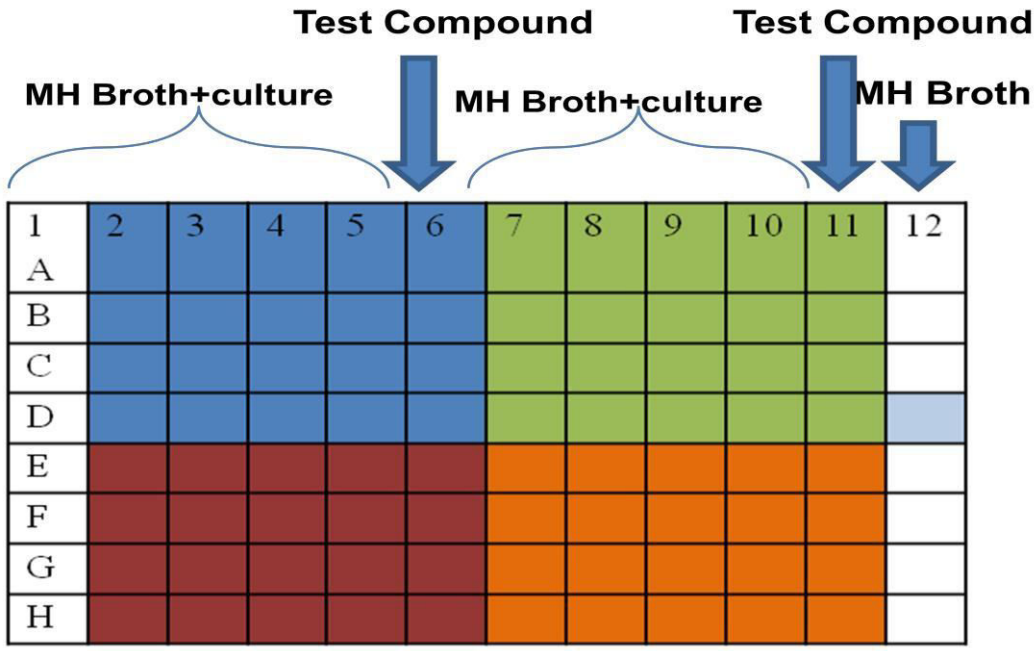


Figure 3.2: Experiment setup for microbroth dilution method

3.2 ANTICANCER STUDIES

3.2.1 MATERIALS

(A) GROWTH MEDIUM FOR CANCER CELL CULTURE

Cell culture is a technique of isolating cells from tissues of animals and humans and maintaining them in controlled artificial conditions. Basic environmental requirements for cells to grow optimally are: controlled temperature, substrate for cell attachment, and appropriate growth medium and incubator that maintains the correct pH and osmolality. Selecting appropriate growth medium for the cell culture is the most crucial and important step for *in vitro* cultivation. Cell culture media is generally composed of all the basic nutrients required by the cell for proliferation and survival. A typical culture medium is composed of a complement of amino acids, vitamins, inorganic salts, glucose, and serum as a source of growth factors, hormones, and attachment factors. The growth medium for cell culture can be divided majorly into two groups:

- **Natural Media**

Natural media is composed of natural biological fluids, for example; serum. This type of media is very convenient to use as it can be used for culturing wide variety of cells. The major disadvantage of natural media is its poor reproducibility due to lack of knowledge of the exact composition of these natural media.

- **Artificial Media**

Artificial or synthetic media are prepared by adding nutrients (both organic and inorganic), vitamins, salts, O_2 and CO_2 gas phases, serum proteins, carbohydrates, cofactors [19].

Artificial media can be divided into four groups:

- **Serum containing media**

Fetal bovine serum or Fetal Calf serum is the most common and important supplement in animal cell culture media. It provides an optimal growth conditions to cells in culture medium. Serum provides carriers or chelators for labile or water-insoluble nutrients, hormones and growth factors, protease inhibitors, and binds and neutralizes toxic moieties.

- **Serum-free media**

Serum has many drawbacks also and this can lead to misinterpretation of results [20] [21]. To overcome this problem, a number of serum-free media have been developed. In this type of media only specific type of cell can be grown and also there is incorporation of defined quantities of purified growth factors, lipoproteins, and other proteins [22]. These media are also referred to as 'defined culture media' since the components in these media are known.

- **Chemically defined media**

The constituents of this type of media are produced in bacteria or yeast by genetic engineering and there is addition of vitamins, cholesterol, specific amino acids, and fatty acids. All these components make this media contamination free with pure protein additives, like growth factors [23] [24].

- **Protein-free media**

Protein-free media do not contain any protein and only contain non-protein constituents. It promotes superior cell growth and protein expression and facilitates downstream purification of the expressed product [25] [26] [27]. Formulations like MEM, RPMI-1640 are protein-free and protein supplement is provided when required.

Basic components of culture medium

- **Buffering systems**

Regulating pH is critical for optimum culture conditions and is generally achieved by one of the two buffering systems:

- Natural buffering system

Gaseous CO_2 balances with the CO_3/HCO_3 content of the media in natural buffering system. These types of cultures are maintained in an air atmosphere with 5% CO_2, in an incubator. They are of low cost and non-toxic [28].

- HEPES

This buffer does not require a controlled gaseous atmosphere because it has a superior buffering capacity in the pH range 7.2- 7.4 [29]. HEPES is relatively expensive and toxic at a

higher concentration for some cell types. HEPES has also been shown to greatly increase the sensitivity of media to phototoxic effects induced by exposure to fluorescent light [30].

- Phenol red

Phenol red is an indicator dye, which is present in most of the commercially available media. It allows constant monitoring of pH. During the cell growth, pH of the media keeps on changing due to release of metabolites by the cells. Phenol red dye helps in the monitoring pH change, as the color of the media changes whenever it becomes acidic or basic. At low pH levels, phenol red turns the medium yellow, while at higher pH levels it turns the medium purple. Medium is bright red for pH 7.4, the optimum pH value for cell culture.

➢ **Inorganic salt**

Inorganic salt in the media help to retain the osmotic balance and help in regulating membrane potential by providing sodium, potassium, and calcium ions [31].

➢ **Amino Acids**

Essential amino acids cannot be synthesized by the cells themselves, therefore they are to be supplemented from outside in the media. They are required for the proliferation of the cells. L-glutamine, an essential amino acid, is particularly important [32]. L-glutamine provides nitrogen for NAD, NADPH and nucleotides and serves as a secondary energy source for metabolism. L-glutamine is an unstable amino acid that is why added in the media just before the use. L-glutamine concentrations for mammalian cell culture media can vary from 0.68 mM in Medium 199 to 4mM in Dulbecco's Modified Eagles's Medium. Nonessential amino acids are also added because during the growth they are depleted.

➢ **Carbohydrates**

Carbohydrates provide energy to the cells and are present in the form of glucose and galactose in the media.

➢ **Proteins and Peptides**

Albumin, transferrin, and fibronectin are the most important proteins and peptides. They are supplied artificially in serum-free media and they are being synthesized by serum in serum-

media. Aprotinin is another important protein which inhibits several proteases such as trypsin.

- **Fatty Acids and Lipids**

They are particularly important in serum-free media as they are generally present in serum.

- **Vitamins**

Group B vitamins are most essential vitamins for the growth and proliferation of cancer cells. They cannot be synthesized by cells themselves, therefore they are to be supplied artificially from outside. Serum is the main source of vitamins.

- **Trace Elements**

Serum- media has some trace elements which are not present in serum-free media. These are to be added in the serum-free media for example: copper, zinc, selenium and tricarboxylic acid intermediates are chemical elements that are needed in minute amounts for proper cell growth [33]. These micronutrients are essential for many biological processes, e.g. the maintenance of the functionality of enzymes.

- **Media Supplements**

Certain cell lines need some additional components in the media, which are usually not present in the basal media. They help sustain proliferation and maintain normal cell metabolism [34 35]. It is recommended to check the osmolality of the media after adding all the components as it can lead to negative effect on the cells. For most of the cell lines, optimal osmolality should be between 260 mOSM/kg and 320 mOSM/kg.

- **Antibiotics**

Antibiotics are added in the media to avoid the bacterial and fungal growth that can cause contamination otherwise and also causes the cell death. Routinely, antibiotics are not used in media because; antibiotics can interfere with the metabolism of sensitive cells.

- **Serum in Media**

It is a complex mixture of albumins, growth factors and growth inhibitors. Serum plays an important role in cell culture media. It serves as a source for amino acids, proteins, vitamins,

carbohydrates, lipids, hormones, growth factors, minerals, and trace elements. Serum from fetal and calf bovine sources are commonly used to support the growth of cells in culture [36]. Fetal serum is appropriate for cell cloning and for the growth of fastidious cells [37]. Calf serum is used in contact-inhibition studies because of its lower growth-promoting properties. 2- 10 % serum is present in normal media.

❖ McCOY'S MEDIA

McCoy's media was used in this project work for the culture of cancer cell lines. The first medium was developed in 1955. The original formulation was based on the amino acids in concentrations similar to those in Eagle's medium as well as the water soluble vitamins of Medium 199. Modifications to the original formulation resulted in the final version which was published in 1960.

	mg/l
INORGANIC SALTS	
Magnesium sulphate anhydrous	97.720
Potassium chloride	400.000
Sodium chloride	6460.000
Sodium phosphate monobasic anhydrous	1220.00
AMINO ACIDS	
Glycine	7.510
L- Alanine	13.360
L- Arginine hydrochloride	42.140
L- Asparagine	45.030
L- Aspartic acid	19.970
L- Cysteine hydrochloride	31.500
L- Glutamic acid	22.070
L- Glutamine	219.200

L- Histidine hydrochloride monohydrate	20.960
l- Hydroxyproline	19.670
L- Isoleucine	39.360
L- Leucine	39.360
L- Lysine hydrochloride	36.540
L- Methionine	14.920
L- Phenylalanine	16.520
L- Proline	17.270
L- Serine	26.280
L- Threonine	17.570
L- Tryptophan	3.060
L- Tyrosine Disodium salt	26.100
L- Valine	17.570
VITAMINS	
Ascorbic acid	0.5625
Biotin	0.200
Choline chloride	5.000
D-Ca-pantothenate	0.200
Folic acid	10.000
Niacin	0.500
Niacinamide	0.500
Pyridoxal hydrochloride	0.500
Pyridoxine hydrochloride	0.500
Riboflavin	0.200
Thiamine hydrochloride	0.200

Vitamin B12	2.000
i-inositol	36.000
p-amino benzoic acid others	1.000
D- Glucose	3000.000
Glutathione reduced	0.500
Peptic digest of animal tissue	600.000
Phenol red sodium salt	11.000

Table 3.1: Composition of McCoy's Media

A **B**

Figure 3.3: **(A)** McCoy's Powdered Media (commercially available, source: Sigma), **(B)** McCoy's Liquid media (prepared in lab)

(B) OTHER MATERIALS

❖ Reagents and Chemicals

DPBS (Dulbecco's Phosphate Buffer saline), McCoy's media, 10 X PBS (Phosphate Buffer saline), 10 X Trypsin, DMSO (Dimethyl sulfoxide), Methanol, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Propidium idodide staining solution, Doxorubicin (5 mM), 5- Fluororacil (10 mM), RNase.

❖ Glassware and Apparatus

Media filteration unit, 96- well and 6- well plates, Multichannel pipette, 25 cm^2, 75 cm^2, 175 cm^2 culture flasks, Glass steripipettes, Falcon tubes (50 ml and 15 ml), eppendorfs, Haemocytometer, FACS tubes (5 ml round bottom), vortex.

❖ Bacterial Cultures and Cancer Cell lines

There are many different types of cancer cell lines on which the anticancer studies are done. The cell lines are specific for specific cancer. There are breast cancer cell lines, colon cancer cell lines, skin cancer cell lines etc. HCT 116 wild type is a colon cancer cell line. The cells are being isolated from the tissues of part of colon having cancer.

HCT 116 p53 -/- cell line was established by knocking out the Tp53 gene which encodes the p53 protein in humans.

Human Colon cancer cell lines HCT 116 wild type and HCT 116 p53-/- (knockout) were used in anticancer cytotoxicity studies.

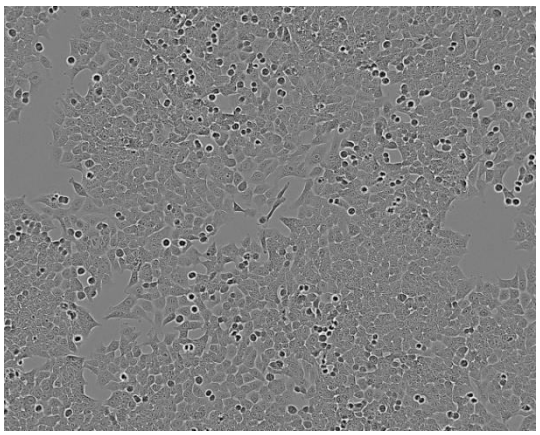


Figure 3.4: HCT 116 cell line

3.2.2 METHODS

The methods that were used in the anticancer studies in this project were:

(A) MTT ASSAY

A number of approaches to quickly and properly evaluate cytotoxicity *in vitro* have been developed. For quantitation of cell viability, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay is the most frequently used. MTT assay measures the conversion of MTT into purple-coloured MTT formazan by the redox activity of living cells, and a decrease in cellular MTT reduction could be an index of cell damage. Viable cells are used in this assay and treated with the test compound in increasing concentration. After 48 hrs incubation, cells are treated with MTT and then solublizing solution is added to measure the cytotoxicity through spectrophotometer.

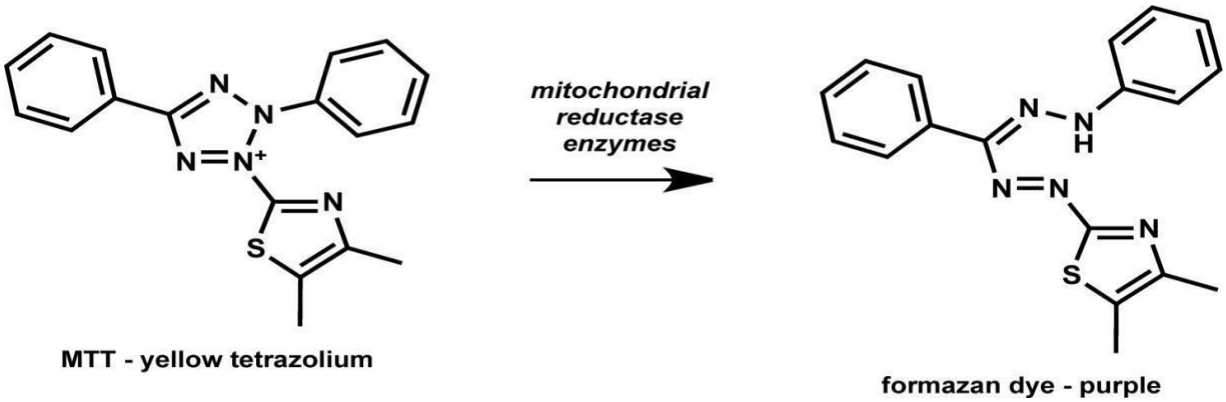


Figure 3.5: MTT reaction

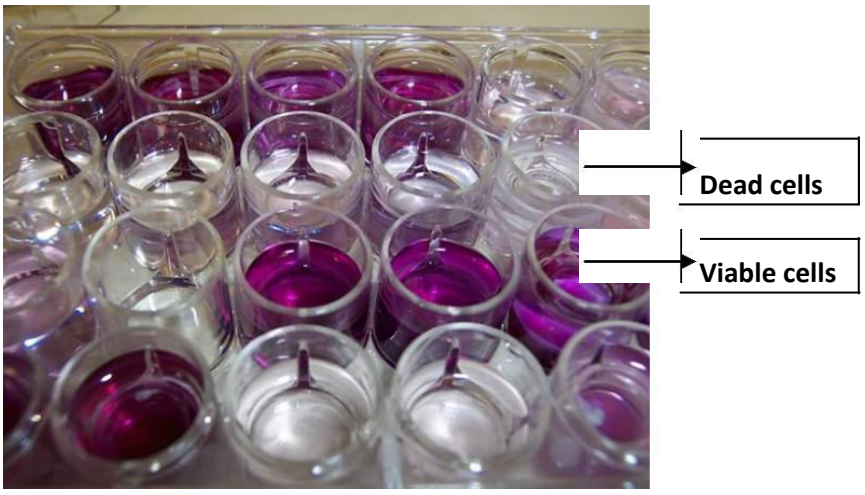


Figure 3.6: MTT assay plate

- **Preparation of media**: McCoy's media was prepared by dissolving the commercially available media powder in 1 l of distilled, autoclaved water inside the laminar airflow bench. It was then filtered using the media filteration unit. 10 ml antibiotic was added in it along with 100 ml of FBS (Fetal Bovine Serum). Media was stored in refrigerator.

- **Thawing the cell lines**: Cryopreserved Colon cancer cell lines HCT 116 wild type and HCT 116 p53-/- were thawed and cultured in 25 cm^2 culture flasks.

- **Subculturing the cell lines:** The cells were subcultured till they become confluent and there are enough cells for the experiment.

- **Preparation of MTT**: 200 mg of MTT powder was weighed and dissolved in 50 ml of MQ water or 10 X PBS. It was then filtered with the help of 0.2 µm syringe filter inside the laminar airflow.

- **Preparation of solubilizing solution**: DMSO and Methanol were mixed in 1:1 ratio to prepare the solubilizing solution.

- **Test protocol**:

    - Confluent flasks of both the cell lines were taken and media was removed.
    - Cells were washed with DPBS to remove any debris.
    - Trypsin (protease) was added in each flask and kept in incubator for 10 min.
    - Cells detached from the surface of the flask were washed with DPBS and collected in the falcon tube.
    - They were then centrifuged at 4,000 rpm for 5 min.
    - Pellet was resuspended in 10 ml of media and cell counting was done on the heamocytometer.
    - Calculations were done and cell suspension was diluted in media so that each well has 5000 cells in 96- well plate.
    - 200 µl of cell suspension media was added in each well. Plate was kept for overnight incubation in the CO_2 incubator.

- Next day, the media was removed and treatment of test compounds along with the control molecules was given in each plate in increasing concentrations of 1 µM, 5 µM, 10 µM, 25 µM, 50 µM.
- Plates were incubated for 48 hrs in CO_2 incubator.
- After that 20 µl of MTT was added in each well and plates were incubated for 4 hrs.
- Media was removes and solubilizing solution was added 200 µl in each well.
- Readings were taken in spectrophotometer at 540 nm.
- Data analysis was done.

Same experiment was done with all the series of all modules.

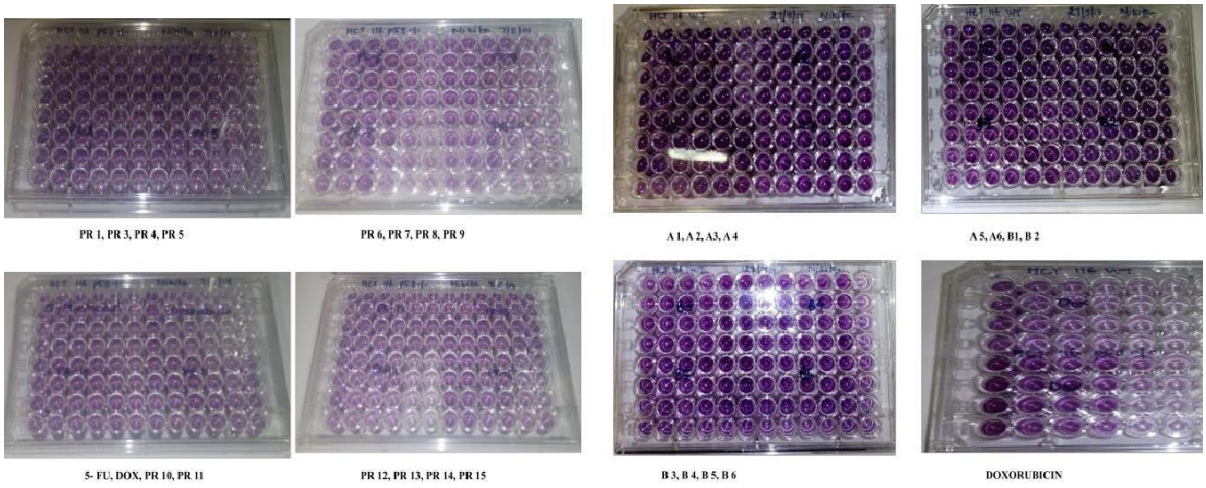


Figure 3.7: MTT assay plates of Module II and Module III

(B) FACs

Fluorescence activated cell sorting is al laser based technique and is a biophysical technology which used for cell sorting, cell counting, biomarker detection and protein engineering. Multiparametric analysis of the physical and chemical characteristics of thousands of cells is possible through this technique. It is a rapid and reliable method to quantify viable cells in a cell suspension. Cell viability determination is critical when a response of cytotoxic drug or molecule is to be tested. Exclusion of dead cell from the experiment is also important and before that, it is necessary to determine the population/number of dead cell in the suspension. Dead cells can uptake fluorescent probes and generate some unwanted results.

For determining the cell death/apoptosis in cell cycle, FACS is used and Propidium iodide (PI) is a widely used membrane impermeant dye that is generally excluded from viable cells.

It intercalates between the base pairs and thus, binds to DNA. PI is excited at 488 nm and, with a relatively large Stokes shift, emits at a maximum wavelength of 617 nm. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) are other fluorochromes which excite at 488 nm and can be used in combination with PI. In this method, cells are seeded in plate and treated with the anticancer molecules. Fixation of cells is done with help of 70% alcohol and then fluorochrome (PI) is added in dark and different phases of cell cycle were analysed using Flow cytometer (BD FAC Verse)

- All the basic preparations for the experiment were same as that of MTT assay.
- Test protocol:

  - Confluent flasks of both the cell lines were taken and media was removed.
  - Cells were washed with DPBS to remove any debris.
  - Trypsin (protease) was added in each flask and kept in incubator for 10 min.
  - Cells detached from the surface of the flask were washed with DPBS and collected in the falcon tube.
  - They were then centrifuged at 4,000 rpm for 5 min.
  - Pellet was resuspended in 10 ml of media and cell counting was done on the heamocytometer.
  - Calculations were done and cell suspension was diluted in media so that each well has 2 X 10^5 cells in 6- well plate.
  - 1 ml of cell suspension media was added in each well. Plate was kept for overnight incubation in the CO_2 incubator.
  - Next day, the media was removed and treatment of test compounds along with the control molecules was given in each plate at 25 µM and 50 µM concentrations.
  - Plates were incubated for 48 hrs in CO_2 incubator.
  - Media was collected in tubes and centrifuged at 5000 rpm for 5 minute. Supernatant was discarded and pellet was resuspended in 1 ml 1X PBS.
  - Centrifuged at 5000 rpm for 5 minutes and process was repeated thrice.
  - 1 ml 70 % ethanol was added in each tube and kept in ice for 2 hours.
  - Again centrifuged at 5000 rpm for 5 minutes and washing with 1X PBS was repeated thrice.

- Pellet was then resuspended in PBS and 2.5 µl RNase was added in each tube.
- Tubes were kept at 37^{o} C in heat block for 40 minutes.
- Suspension was transferred in FACs tubes and Propidium iodide was added and kept for 10 minutes.
- FACs analysis was done with PI filter.

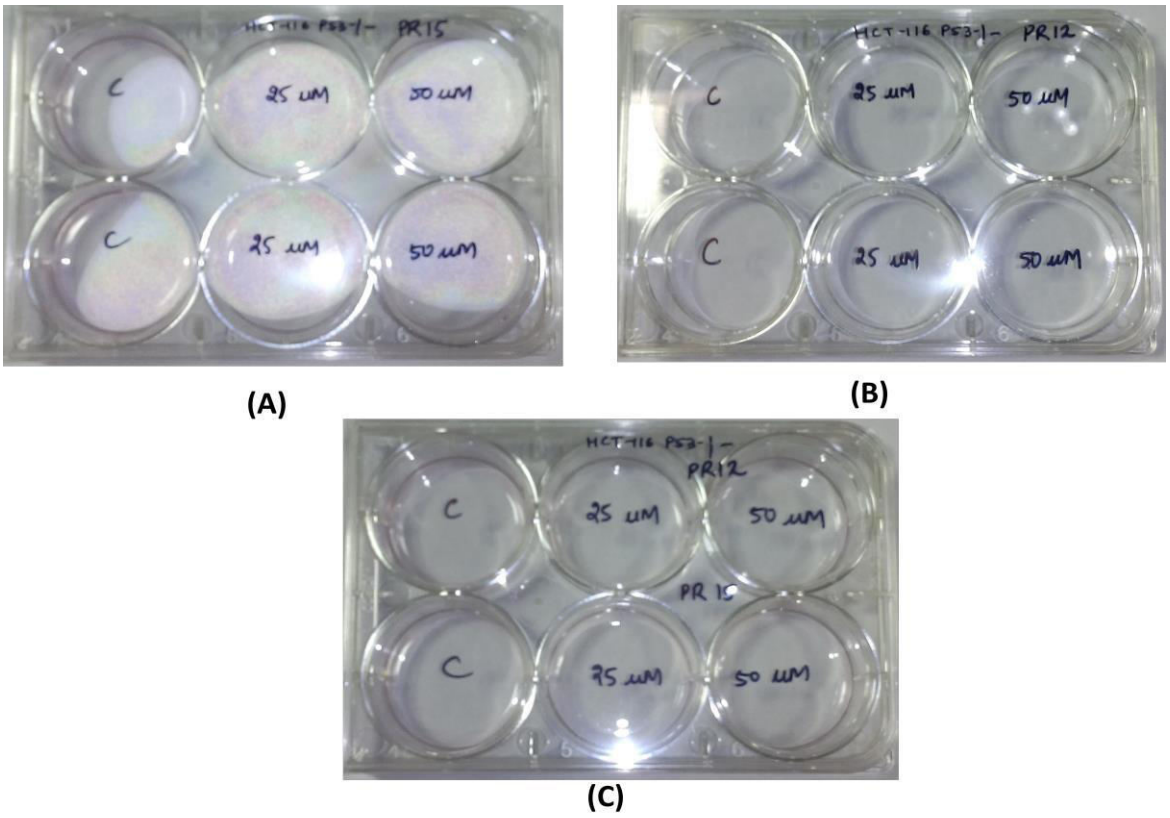


Figure 3.8: 6- well plates containing trypsinised cells for cell cycle; (A) PR 15 (B) PR 12 (C) PR 15 and PR 12

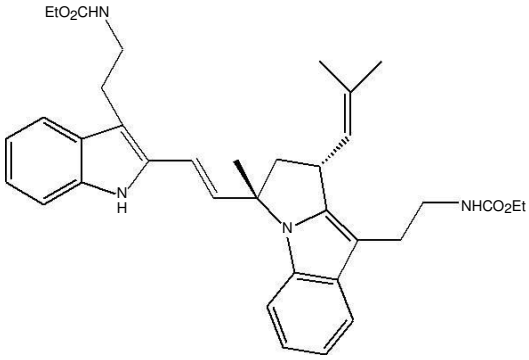
3.3 TEST COMPOUNDS

For antibacterial and anticancer screening, different types of methods were used. There were three different modules in which same type of methods were used. Each module had different series of molecules.

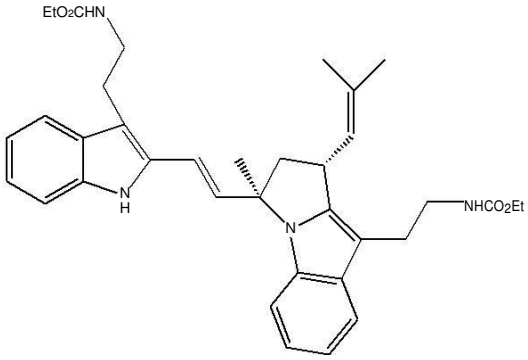
3.3.1 MODULE 1

There were two different series of molecules in this module, on which both antibacterial and anticancer studies were performed.

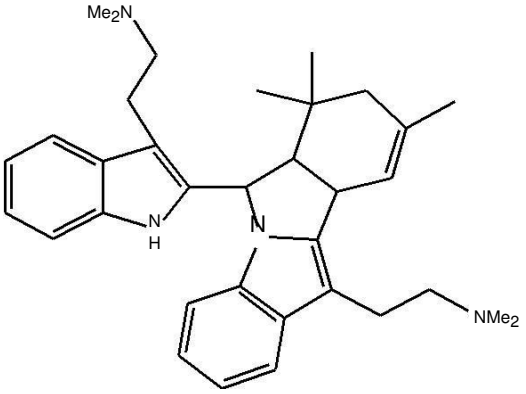
SERIES- I



672 C



672 B



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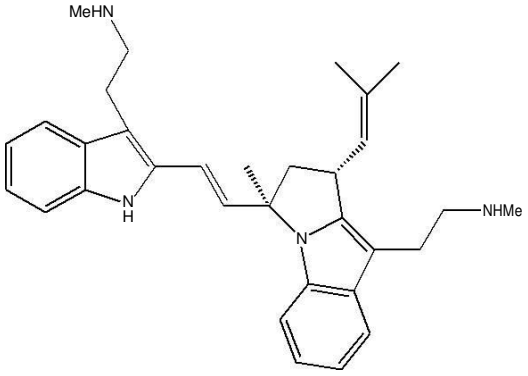
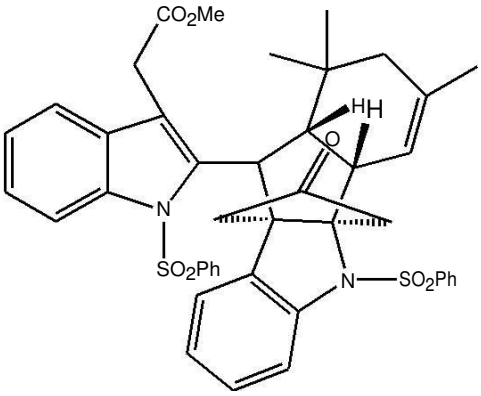
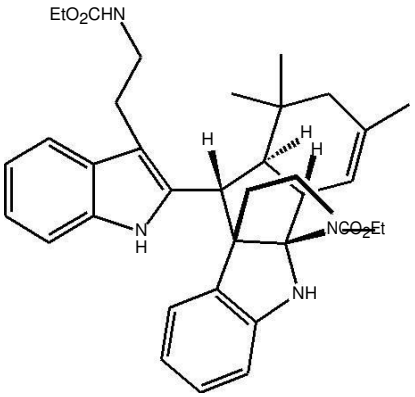


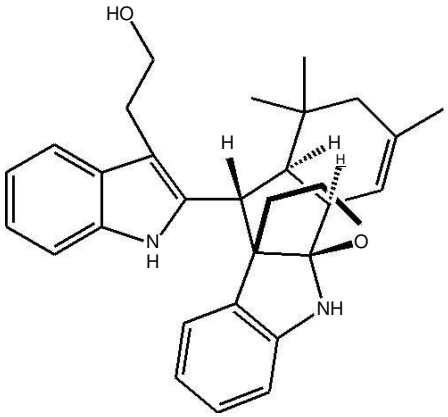
FLINDEROLE A



819



834 NP



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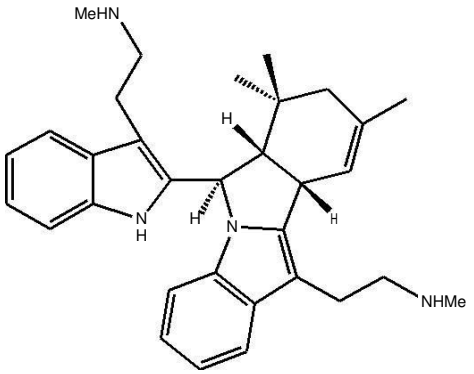
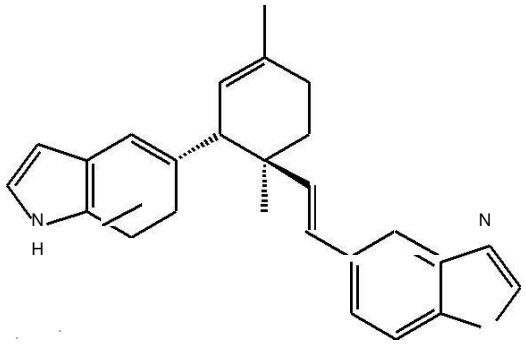
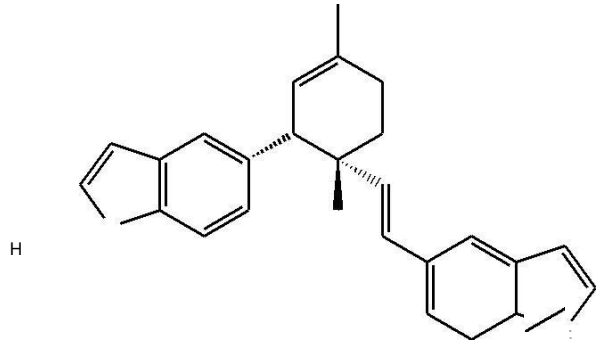


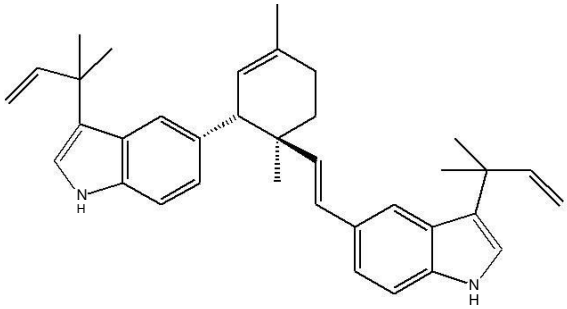
ISOBORREVERINE



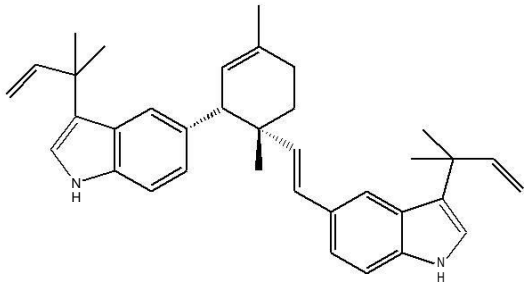
CAULI- A



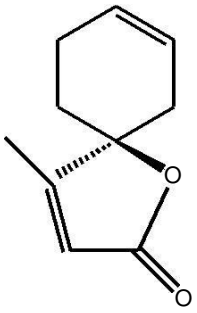
CAULI -B



CAULI- C

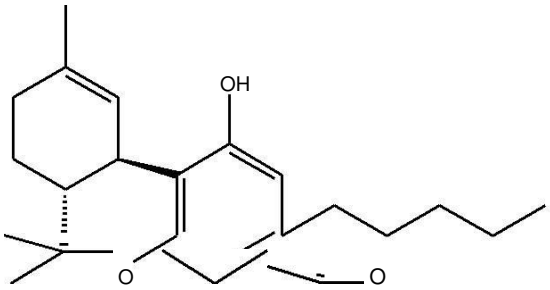


CAULI- D

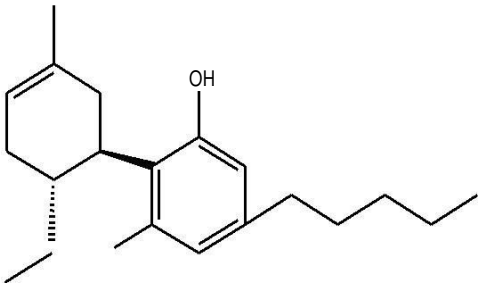


ANDI-A

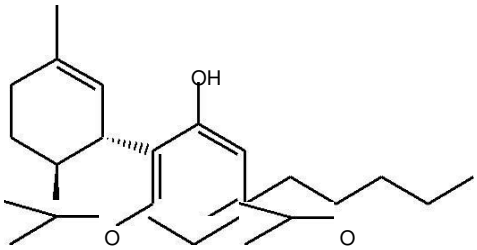
SERIES- II



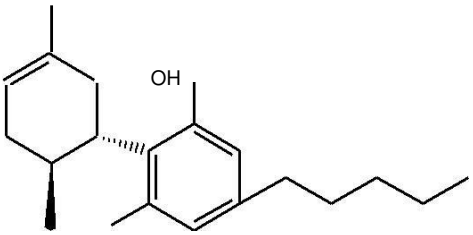
SM-1



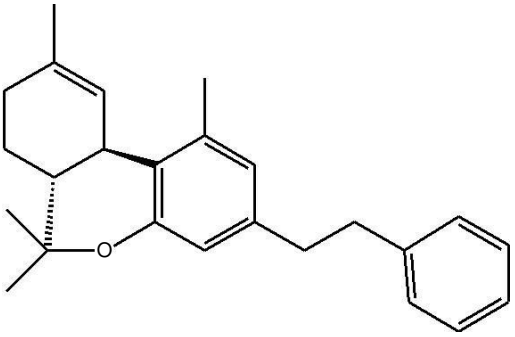
SM-2



SM-3



SM-4



SM-5

Among all these compounds, Flinderole–A and Isoborreverine were used as control compounds. Isoborreverine and Flinderole A were isolated from ***Flindersia acuminata*** and they both are alkaloids. Flinderoles are identified as having good antimalarial activity whereas Isoborreverines are antiparasitic in nature. Flinderole A and Isoborreverine were chemically synthesized with modifications of different head groups. *Flindersia acuminate* belongs to Rutaceae family. Commonly it is known as Icewood; Maple, Silver; Paddy King's Beech; Pine, Putt's; Putt's Pine; Silver Silkwood; Silver Maple; White Silkwood; Silkwood, Silver. Its bark is usually pale brown. Stem is inconspicuous and pale brown stripes are

usually visible in the outer blaze. It is very well known plant because of its medicinal properties through flinderoles and isoborreverine.

A **B**

Figure 3.9: (A) Leaves and fruit **(B)** Crown flower of *Flindersia acuminate*

3.3.2 MODULE 2

This module consisted of only one series, which included 15 compounds. The chemical structure of these compounds was not provided. Chemical formulae were given only along with their molecular weights. 10 mm stock concentration of each compound was prepared. All the compounds were soluble in DMSO.

Sample code	Molecular Formula
PR-1	$C_{34}H_{23}N_3O_2$
PR-3	$C_{34}H_{22}BrN_3O_2$
PR-4	$C_{33}H_{21}N_3O$
PR-5	$C_{34}H_{23}N_3O$
PR-6	$C_{33}H_{20}BrN_3O_2$
PR-7	$C_{33}H_{20}ClN_3O_2$
PR-8	$C_{34}H_{22}ClN_3O_2$
PR-9	$C_{33}H_{19}BrClN_3O_2$
PR-10	$C_{34}H_{23}N_3O$
PR-11	$C_{35}H_{25}N_3O$
PR-12	$C_{34}H_{22}BrN_3O$

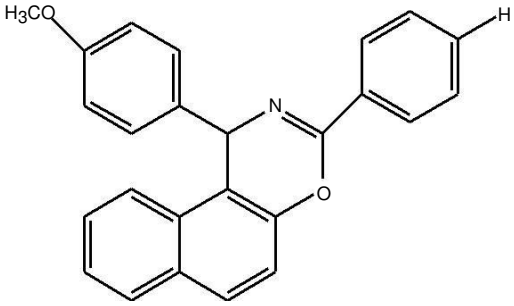
PR-13	$C_{35}H_{25}N_3O_3$
PR-14	$C_{34}H_{23}N_3O_2$
PR-15	$C_{34}H_{22}ClN_3O_2$
PR-16	$C_{35}H_{25}N_3O_2$

With this set of molecules/compounds, Doxorubicin and 5-Fluorouracil were taken as control to check the anticancer properties. Antibacterial screening of these compounds were not performed. Only anticancer screening was done.

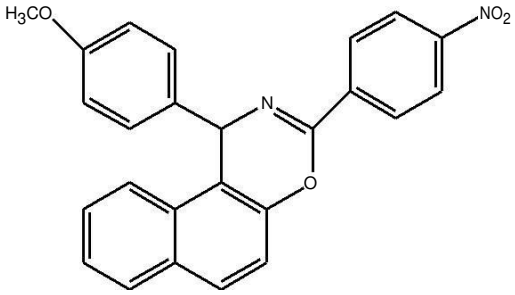
3.3.3 MODULE 3

This module consisted of two different series which included 12 compounds. The basic structure moiety was same of all the compounds; the main difference was alkyl groups. The structures of all the compounds are as follows:

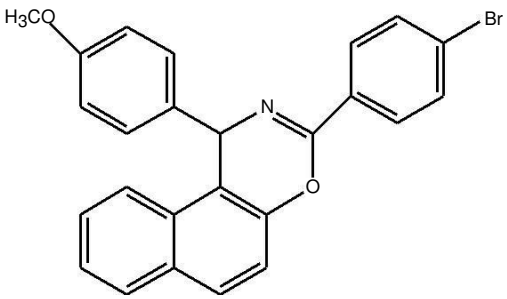
SERIES-I



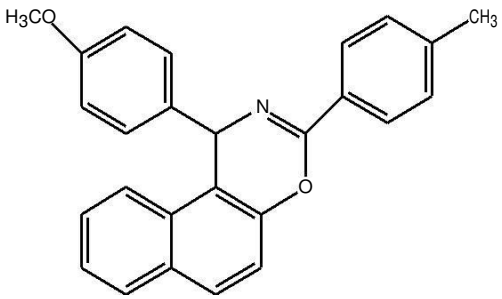
A1



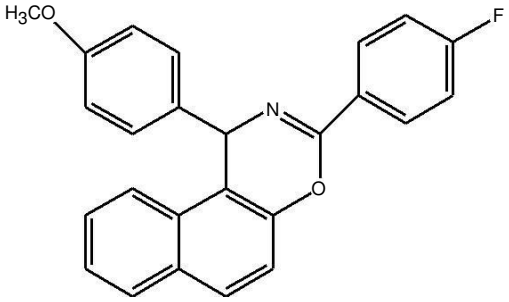
A2



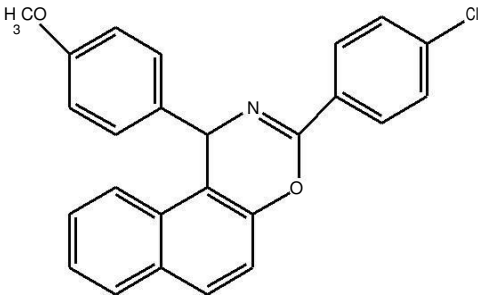
A3



A4

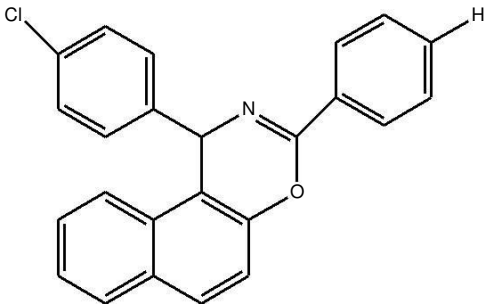


A5

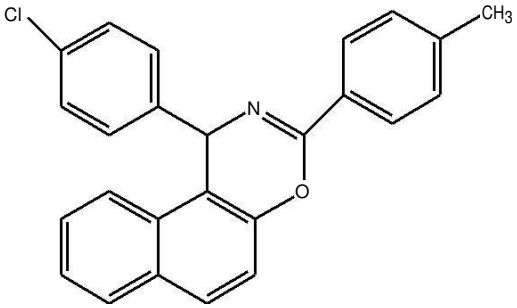


A6

SERIES II



B1



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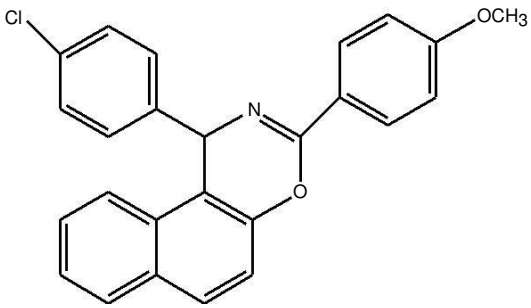

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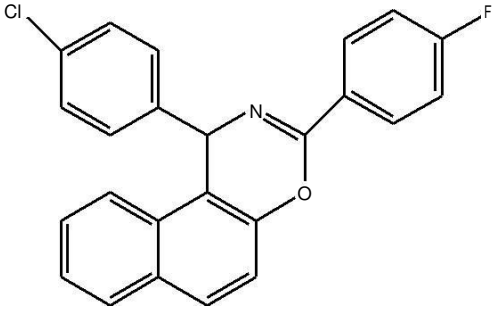
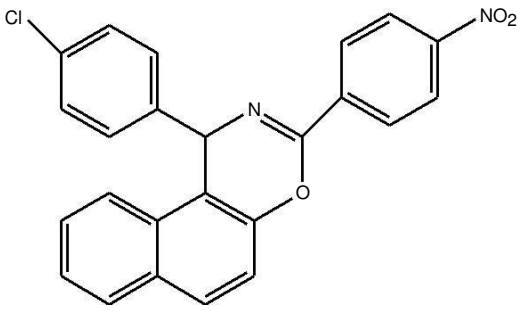
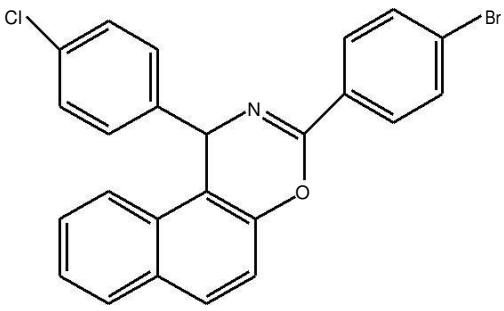


B4



B5



B6

With this group of compounds, same type of experiment was done. 10 mM concentration of stocks was prepared. Doxorubicin was taken as control along with all these molecules. Antibacterial screening was not performed with these molecules, only anticancer studies were done.

CHAPTER 4
RESULTS

4.1 RESULTS FROM ANTIBACTERIAL SCREENING

(A) MICROBROTH DILUTION RESULTS

Antibacterial screening was done with 18 molecules of Series I and Series II of Module 1. None of the compound was found to be an active antibacterial. Turbidity was seen in all the wells of the 96- well plate and OD was significantly high. As compared to control, none of the compound showed any activity on both the bacterial strains.

(B) COLONY FORMING UNIT COUNT

10^{-1} to 10^{-8} dilutions were made and plated. Inoculum was highly concentrated and had a number of cells. Therefore, there were lawns of microbial cultures on all the concentrations and some of them were too numerous to count (TNTC).

4.2 RESULTS FROM ANTICANCER SCREENING

(A) MTT RESULTS

Following are the results and discussions of the MTT assay performed on various molecules for cytotoxicity studies.

❖ **MODULE 1**

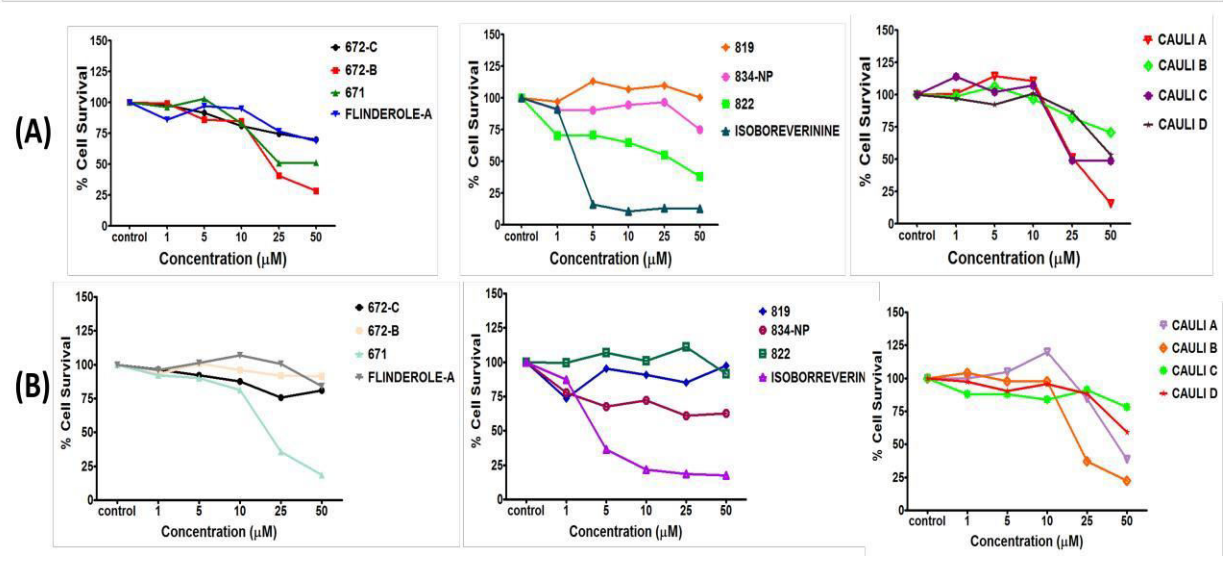


Figure 4.1: Graphs of cytotoxicity in HCT 116 (**A**) wild type cells and (**B**) p53-/- after treatment with Series I of Module I at 1 µM, 5 µM, 10 µM, 25 µM, 50 µM concentrations.

Molecules	IC_{50} µM HCT 116 p53 -/-	IC_{50} µM HCT 116 WT	Molecules	IC_{50} µM HCT 116 p53 -/-	IC_{50} µM HCT 116 WT
672 C	--	--	**FLINDEROLE A**	--	--
672 B	--	21.78	**ISOBORREVERINE**	3.88	3.28
671	20.11	25.32	**CAULI A**	43.72	26.11
819	--	--	**CAULI B**	21.88	--
834 NP	--	--	**CAULI C**	--	24.83
822	--	32.51	**CAULI D**	--	--

Table 4.1: IC_{50} values of Series I of Module I

Isoborreverine was used as a control, but from the previous reports in literature it is having antiparasitic potential. In the current screening it has shown anticancer activity in HCT 116 wt as well as in HCT 116 p53-/- cell lines. Tp53 is a gene that encodes for the p53 protein, which is a tumor suppressor gene; due to presence of p53 gene, cells do not proliferate uncontrollably. It is a potent inhibitor of cell growth and transformation. To study the mechanism of tested molecules, we have used p53-/- (knockout) cell lines of colon cancer HCT-116 p53-/-. From this cell line, p53 gene was removed, because of absence of p53, cells grow uncontrollably. To study the p53 dependent/independent mechanism of any molecule under investigation, cytotoxicity assay was performed with same molecules in the wild type and p53 null cell line of colon cancer and marked variation has been observed, in few of the molecules we have seen difference in IC_{50} values. In this series, Isoborreverine, Cauli B, 671 molecules have shown difference in IC_{50} value in p53 null cell lines. These results indicate in Cauli A, IC_{50} values were higher in p53 knockout cells as compared to HCT-116 WT cells and there is a 2 fold difference. This shows the p53-/- dependent mechanism of Cauli A in HCT 116 cells. Which can be further explored to study the mechanism of this molecule. Similarly, molecules 672 B, 822 and Cauli C were active in HCT 116 WT cells where as Cauli B was found to be active in only HCT-116 p53-/- cells. So it can be hypothesized on the basis of these observations that molecules which are active in only HCT-116 WT cell i.e 672 B, 822 and Cauli C goes by p53 dependent mechanism. Cauli B which is active in only HCT-116 p53-/- cells also acts by p53 dependent mechanism. But few alternative mechanisms are also playing a major role in the process which needs to be investigated further.

❖ MODULE 2

This module consisted of 12 molecules. Doxorubicin was taken as control along with these molecules. The concentrations of Doxorubicin that were tested are µM, 5 µM, 10 µM, 25 µM, 50 µM. The cytotoxic graphs obtained in different cell lines are as follows:

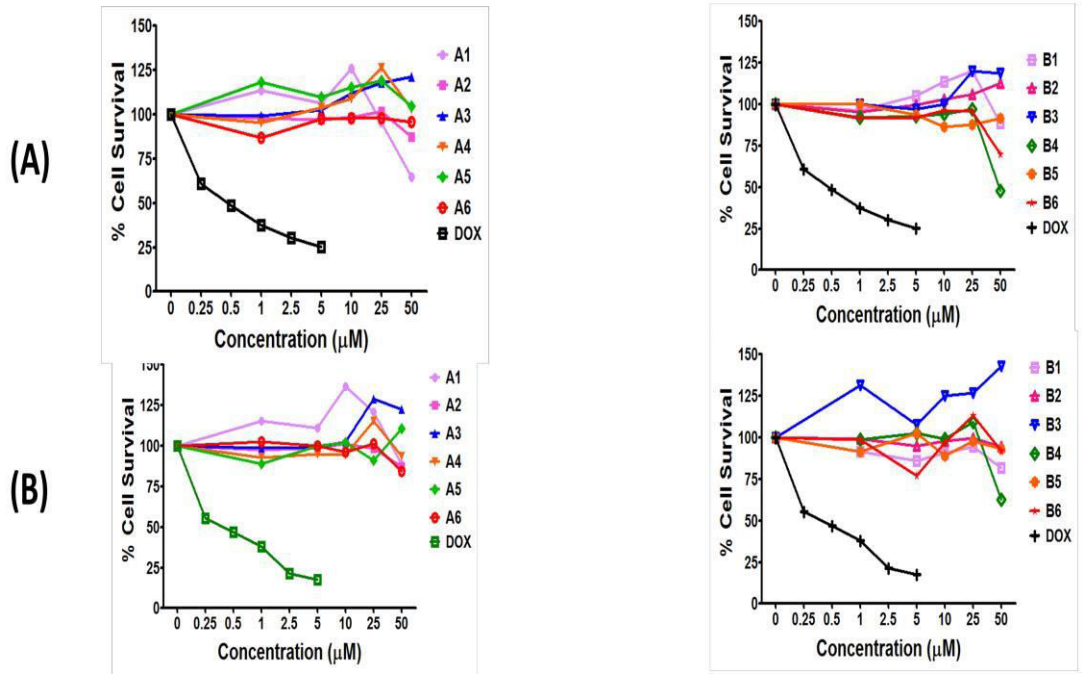


Figure 4.2: Graphs of cytotoxicity in HCT 116 (**A**) wild type and (**B**) p53-/- cells after treatment with Series I and Series II of Module II at 1 µM, 5 µM, 10 µM, 25 µM, 50 µM concentrations.

Molecules	IC_{50} µM HCT 116 p53 -/-	IC_{50} µM HCT 116 WT	Molecules	IC_{50} µM HCT 116 p53 -/-	IC_{50} µM HCT 116 WT
A1	--	--	**B1**	--	--
A2	--	--	**B2**	--	--
A3	--	--	**B3**	--	
A4	--	--	**B4**	--	48.74
A5	--	--	**B5**	--	--
A6	--	--	**B6**	--	--

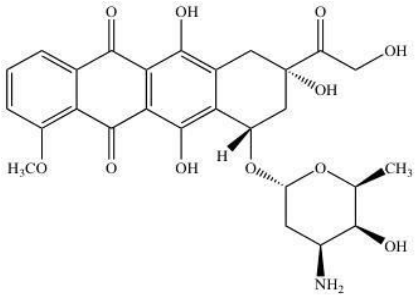
Table 4.2: IC_{50} values of Series I and II of Module II

Molecules present in Module II didn't show any specific activity at the tested concentrations in HCT 116 wild type cells. IC_{50} values were more than the concentration tested.

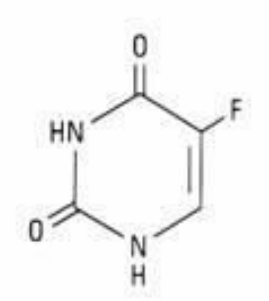
When data from both cell lines was evaluated, it was observed that none of the molecule from this module had any type of anticancer property.

❖ MODULE 3

Doxorubicin and 5- Fluorouracil were taken as controls in this module. 5 mM of Doxorubicin was used in 0.25 µM, 0.5 µM, 1.0 µM, 2.5 µM, 5 µM. 5-Fluororacil was used 10 mM in 1 µM, 5 µM, 10 µM, 25 µM, 50 µM concentrations. Doxorubicin also known as hydroxydaunorubicin, is a drug used in cancer chemotherapy and derived by chemical semisynthesis from a bacterial species. 5- Fluorouracil is a drug that is a pyrimidine analog which is used in the treatment of cancer. it belongs to the family of drugs called the antimetabolites.



Doxorubicin



5- Fluorouracil

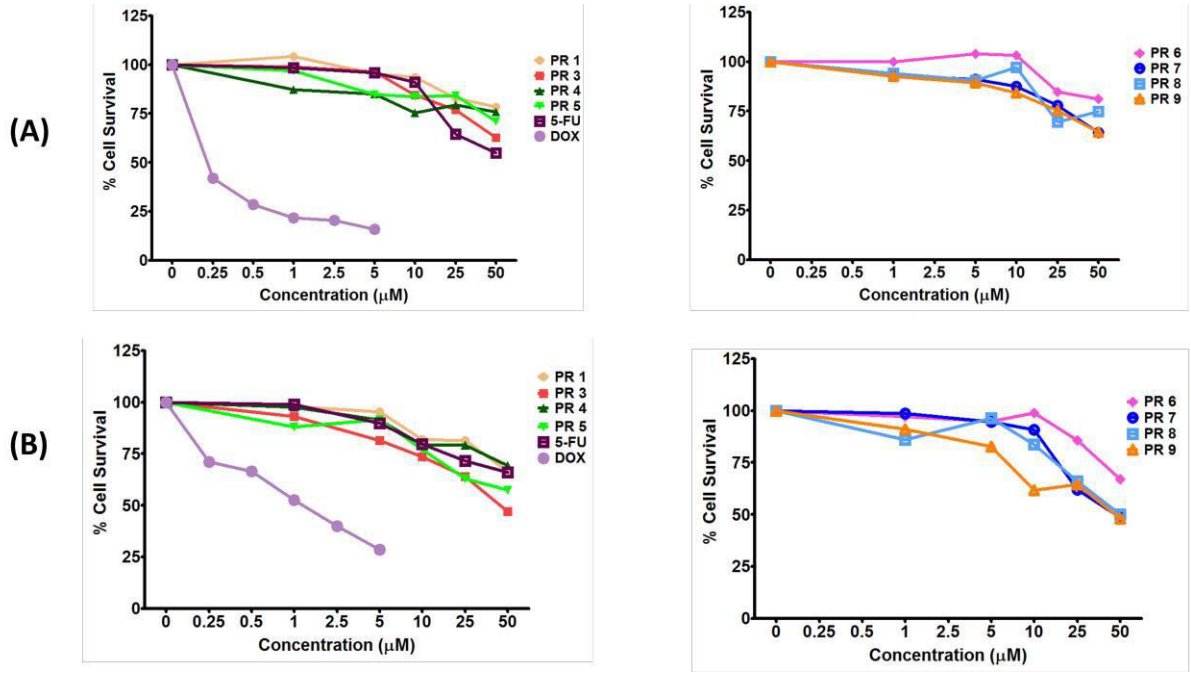


Figure 4.3: Graphs of cytotoxicity in HCT 116 (**A**) wild type and (**B**) p53-/- cells after treatment with Series I of Module III at 1 µM, 5 µM, 10 µM, 25 µM, 50 µM concentrations.

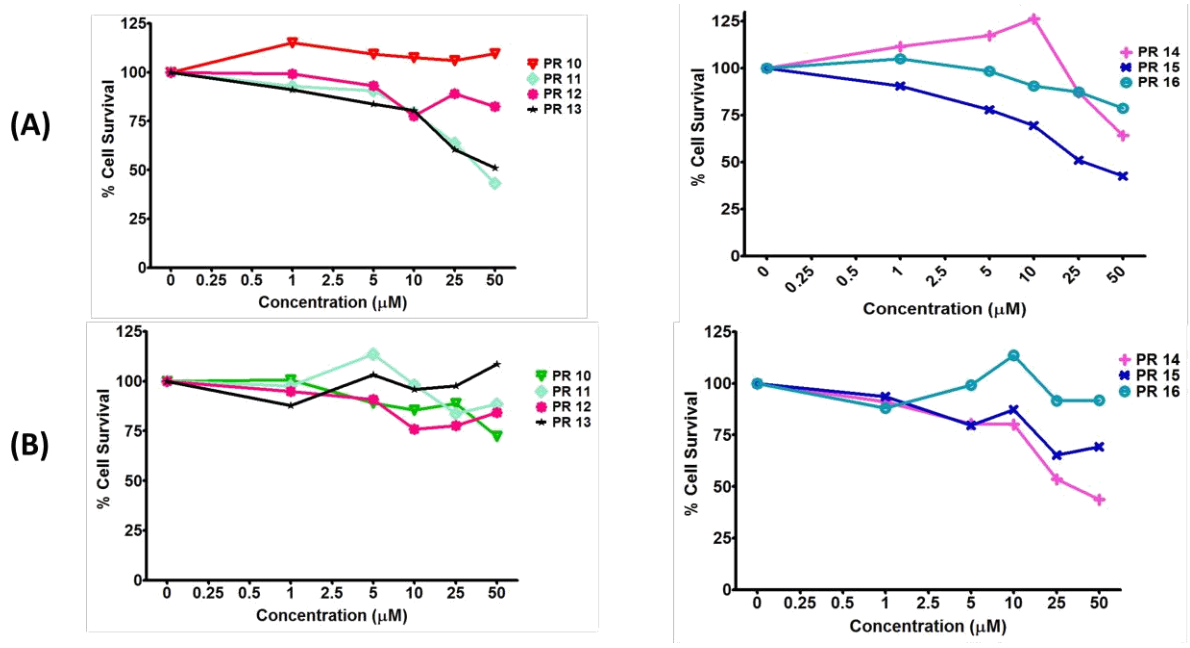


Figure 4.4: Graphs of cytotoxicity in HCT 116 (**A**) wild type and (**B**) p53-/- cells after treatment with Series I of Module III at 1 µM, 5 µM, 10 µM, 25 µM, 50 µM concentrations.

Molecules	IC_{50} µM HCT 116 p53 -/-	IC_{50} µM HCT 116 WT	Molecules	IC_{50} µM HCT 116 p53 -/-	IC_{50} µM HCT 116 WT
PR 1		--	**PR 11**	--	41.66
PR 3	45.49	--	**PR 12**	--	**24.83**
PR 4	--	--	**PR 13**	--	--
PR 5	--	--	**PR 14**	32.51	--
PR 6	--	--	**PR 15**	--	**27.69**
PR 7	47.06	--	**PR 16**	--	--
PR 8	49.73	--	**DOX**	1.32	0.23
PR 9	47.06	--	**5-FU**	--	--
PR 10	--	--			

Table 4.3: IC_{50} values of compounds of Module III

Molecules present in Module III didn't show any specific activity in HCT 116 wild type and HCT 116 p53-/- cells. IC_{50} values were more than the concentration tested. However, two molecules showed some activity and their IC_{50} values were notable, therefore, they were further analysed by cell cycle experiments.

(B) CELL CYCLE ANALYSIS

To explore the fate of cells in different phases of cell cycle, we have performed Propidium iodide based cell cycle analysis in cell lines HCT 116 wild type and HCT 116 p53-/- upon

treatment of PR-12 and PR-15 molecules of Series I of Module III at 25 µM and 50 µM for 48h.

CELL LINE HCT 116 WILD TYPE:

No significant change was observed after treatment in any of phases of cell cycle in HCT 116 wild type cells.

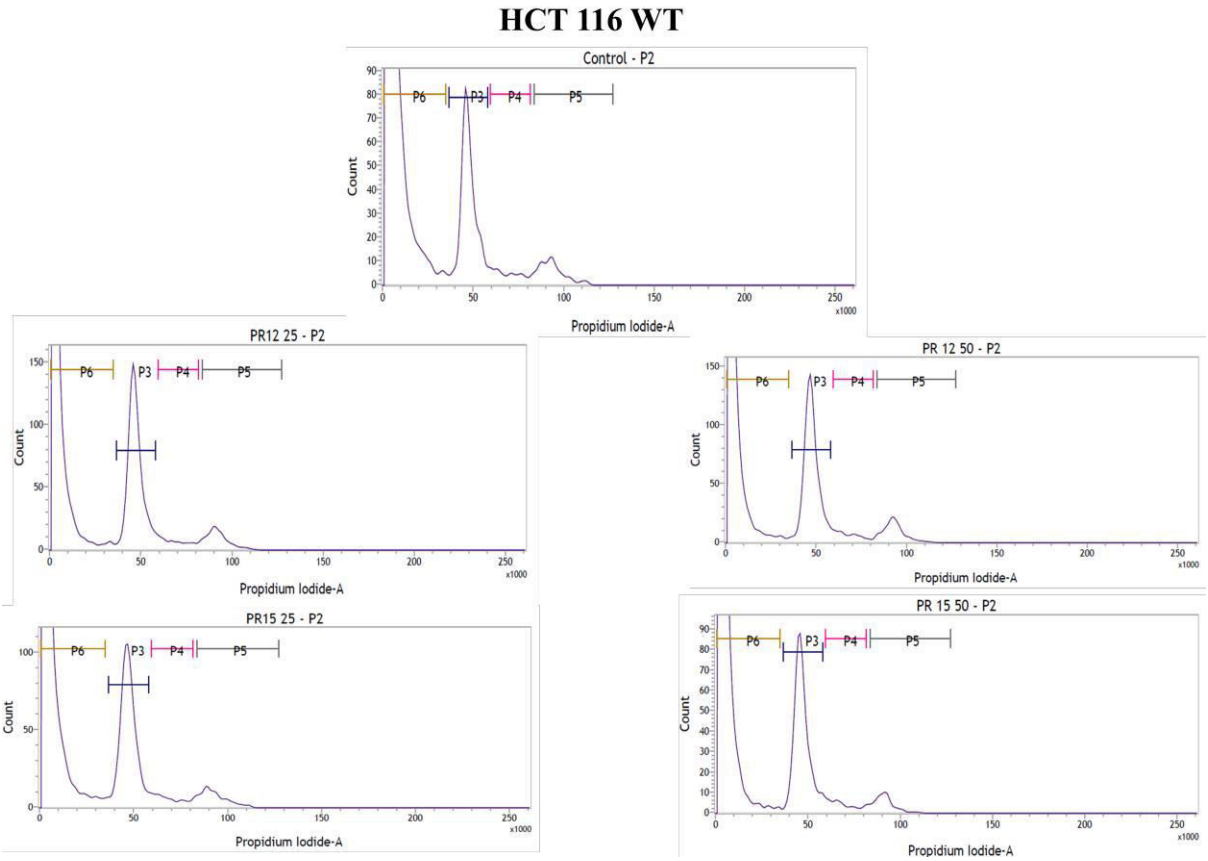


Figure 4.5: Cell cycle histograms of cell line HCT 116 wild type after treatment with PR-12 and PR-15 at 25 µM and 50 µM upto 48h.

CELL LINE HCT 116 p53-/- :

No significant change was observed after treatment in any of phases of cell cycle in HCT 116

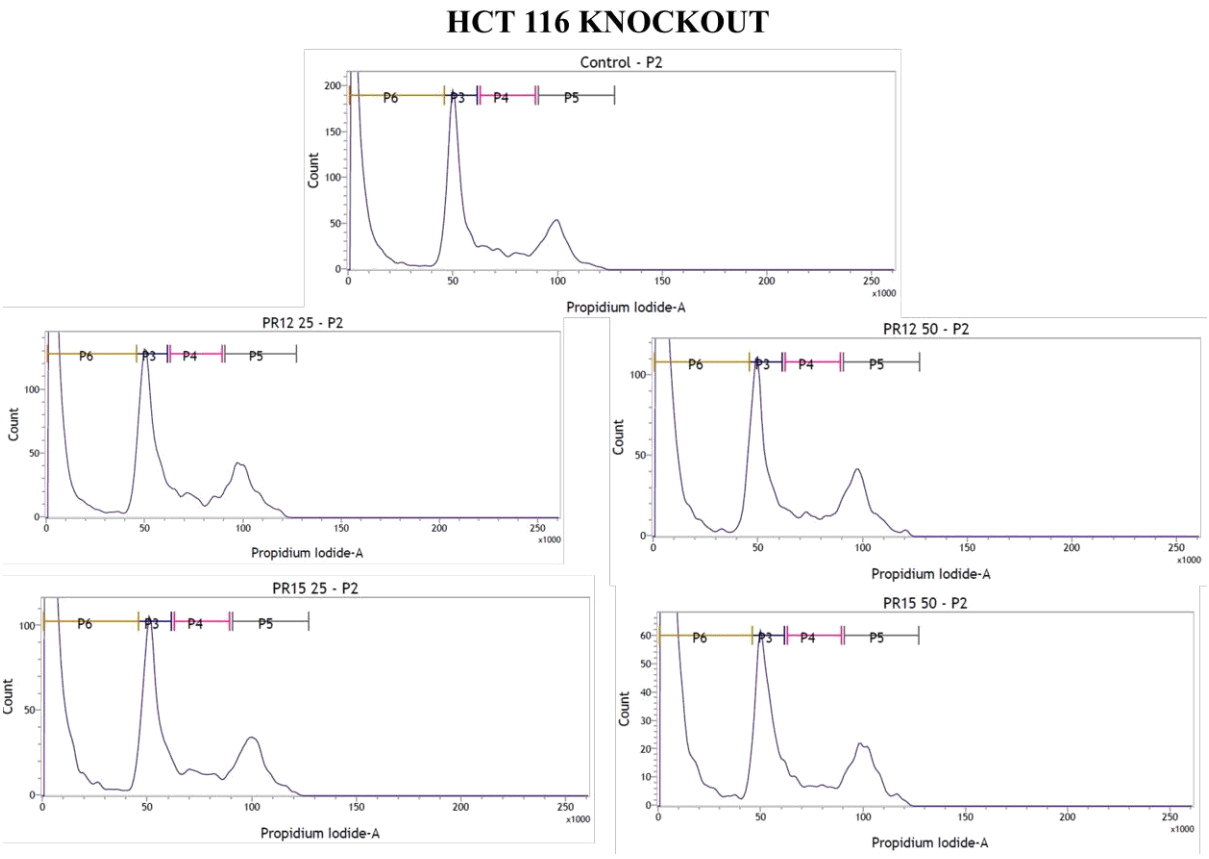


Figure 4.6: Cell cycle histograms of cell line HCT 116 p53-/- after treatment with PR-12 and PR-15 at 25 µM and 50 µM upto 48h.

After cell cycle analysis we didn`t observe any change in different phases of cell cycle as compared to the control cells without any treatment. This indicates that these molecules are not affecting cell cycle at the tested concentration.

CONCLUSION

We have tested different series of molecules for their anticancer and antibacterial potential.

We have tested all the molecules upto 200µM conc. In between we have taken few more concentrations. But none of the molecules have shown any antibacterial activity against the gram negative *E.coli* and gram positive *S.aureus.*

Similarly all the molecules from different series tested in colon cancer cell lines HCT116 WT and p53-/- cell lines. Molecules present in Series I of Module I were showing some promising results as they are seem to be active in cancer cell lines. Isoborreverine is an antiparastic in nature; it showed some activity in both the cell lines. Therefore, it must be following p53 independent pathway in the cell. Similarly, Cauli B and 671 molecules were also showing p53 independent results. Other molecules showed some activity in wild type cell lines. Overall, these molecules can be further studied to know their mechanism of action and the pathway they are following for cell apoptosis. These molecules can proof to be a potent drug lead in future, to develop some drug for cancer therapy. In Module III, we have tested the concentration upto 50µM but only PR12 and PR15 molecules have shown IC_{50} value less than 50µM. 50 % of inhibition was not observed upto maximum concentration of 50 µM. Same molecules were subjected to FACS for cell cycle analysis but didn`t observe any change in different phases of cell cycle.

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