

TARGETING BREAST CANCER USING ZEATIN AND IT'S EFFECT ON CELL PROLIFERATION AND EXPRESSION STUDIES

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

Haritha S (40230012),
Gowthaman J (40230011)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

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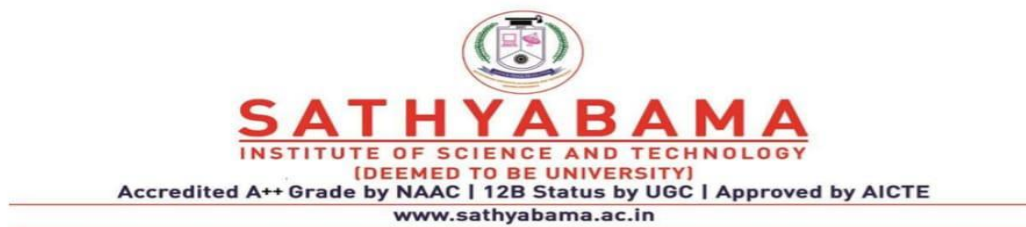
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Haritha Sivakumar (40230012)**, **Gowthaman. J (40230011)** who carried out the project entitled “**Targeting Breast Cancer Using Zeatin and its Effect on Cell Proliferation and Expression Studies**” under our supervision from January 2024 to March 2024

Internal Guide

(Dr. M. BAVANILATHA)

Head of the Department

(Dr. V. RAMESHKUMAR)

Submitted for Viva voce Examination held on 05. 04. 2024

Internal Examiner

External Examiner

DECLARATION

We **HARITHA SIVAKUMAR, GOWTHAMAN.J** hereby declare that the Project Report entitled **“Targeting Breast Cancer Using Zeatin and its Effect on Cell Proliferation and Expression Studies”** done by me under the guidance of Dr. M. Bavanilatha (Internal) is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in **Biotechnology**.

DATE:

PLACE:

SIGNATURE OF THE CANDIDATE

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ABSTRACT:

Breast cancer remains a serious health issue. This study investigates the plant hormone zeatin as a possible tool in the fight against Breast cancer. Zeatin is well recognized for controlling plant growth and development, but new studies indicate it may also have anticancer effects. In order to determine how effective Zeatin is, the study employs two methods: first, it examines the drug's impact on the growth of breast cancer cells in a laboratory setting (*invitro*); second, it uses computer modeling (*insilico*) to determine potential interactions between Zeatin and a protein called Leukemia Factor 4 (LF4), which has been connected to the development of cancer. Researchers will cultivate breast cancer cells in the lab and subject them to various Zeatin concentrations. To find out whether Zeatin suppresses cell growth and whether the effect varies with dosage, methods such as MTT assays will be used to detect the rates at which cells proliferate. They might also look into how Zeatin impacts cell division or causes cell death. The possible mechanism will be further explored using *insilico* modeling. Zeatin's interaction with LF4 will be predicted by this computer simulation. Zeatin may target LF4 to prevent the growth of breast cancer cells if the simulation indicates a strong interaction between the two. The goal of the research is to present a more complete picture of zeatin's potential as a breast cancer treatment by integrating various methods. While the *insilico* modeling will provide insight into the potential molecular target and method of action, the in vitro study will demonstrate the direct impact on cancer cell proliferation. The amalgamated understanding has the potential to facilitate additional exploration and advancement of Zeatin as an innovative treatment approach for breast cancer.

Keywords: Cancer, Breast Cancer, Repurposing drug, Zeatin, EZH4, qRT-PCR.

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LIST OF ABBREVIATIONS

EZH2- Enhancer of zeste homolog 2

HCC- Hepatocellular carcinoma

NK- Natural Killer cells

NSCLC- Non-Small Cell Lung Cancer

EMT - Epithelial-Mesenchymal Transition

NF- κ B - Nuclear factor kappa B

TNF- α - Tumor Necrosis Factor α

IL-6 - Interleukin-6

IL-1 β - Interleukin- 1 β

HeLa - Henrietta Lacks

MDA MB 231 - MD Anderson-Metastatic Breast-231 Cells

DMEM - Dulbecco's Modified Eagle Medium

MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

AO/EB - Acridine Orange/Ethidium Bromide

qRT PCR - Quantitative Real-Time-Polymerase Chain Reaction

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CHAPTER 1

INTRODUCTION

1.1 CANCER:

Cancer manipulates cellular control, which becomes a sneaky approach. Healthy cells divide as necessary and become out of form according to programmed scripts. This balance has been disturbed by malignancy. Cells become restless due to mutations that are triggered by environmental factors or inheritance. They progress into abnormal masses known as tumors; some have boundaries and are harmless, whereas others are deadly and invasive. These malignant antagonists are the purest form of cancer: they proliferate on their own, disregard signals to stop, prevent cell death, replicate endlessly, and then branch out to locations beyond their origin.

Breast cancer predominantly impacts the breast tissue; other subtypes originate in glands, milk ducts, or the overexpression of particular amino acids. Mammograms and diagnostic evaluations, and reflections constitute essential instruments in accomplishing detection at an early stage. Alternatives for treatment encompass the use of radiation that focuses on the tumor, surgery to remove the tumor, and chemotherapeutic to initiate a systemic response. Treatment with hormones treats malignant tumors driven by the hormones estrogen and progesterone, while specialized drugs and immunotherapy enable specialized methods.

The path beyond diagnosis isn't a simple one. Managing both the mental and physiological adverse reactions associated with therapy calls for a comprehensive methodology. While therapy emphasizes psychological consequences, support groups provide consolation and an opportunity for exchanging views. Researchers constantly seek to push the boundaries: advances in immunotherapy harness the effectiveness of the body's immune system, precision healthcare personalized treatments to a tumor's precise composition, and initial identification strives for ever-higher accuracy. In the end, treating cancers requires collaboration; enhanced information, evolving therapies, and

persistent investigation provide an indication of opportunity fighting this powerful disease.

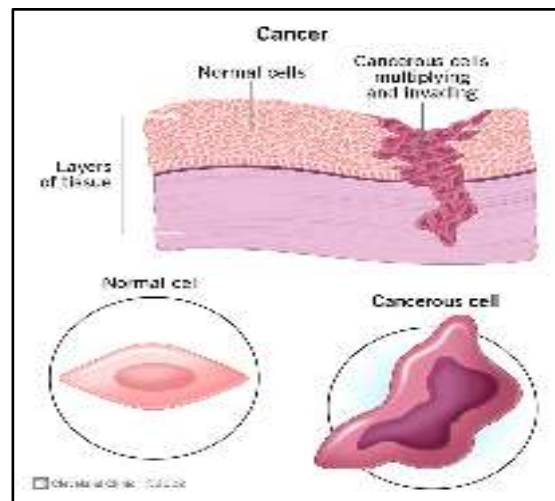


Figure 1.1 Normal and Cancer Tissue

1.2 STAGES IN CANCER:

Cancer staging determines the severity of a cancer, impacting the course of action of therapies and prognosis. Consider an organization comprising groups, usually ranging from stages I to IV. The initial phase has been defined by an insignificant, confined tumor, which is the first competitor. In stages II and III, the threat is more dangerous; the tumor could grow more extensive and can penetrate the surrounding tissues and lymph nodes, but it is uncommon to make its way to organs that are far away. The most serious stage, IV, indicates cancer metastases, or the spread of the cancer to distant organs. The basic idea of staging systems is still unchanged irrespective of the type of cancer: it's essential to know how far the condition has progressed.

Alterations in healthy cells triggered by external contaminants that are either carcinogens or inheritance. The expansion regulation of the cell is harmed by these genetic changes, creating the cells independent agents. Tumors are anomalous clumps that can penetrate adjacent tissues due to growth that is uncontrolled. The most terrible twist is the fact that certain cancers can become able to continue spreading; fragmented cancer cells can use

the body's highways to generate new tumors in locations far away. Understanding these stages of amplification gives us crucial information to respond; early detection through screens and evaluations of themselves becomes vital, enabling the greatest possibility for overcoming this powerful attacker.

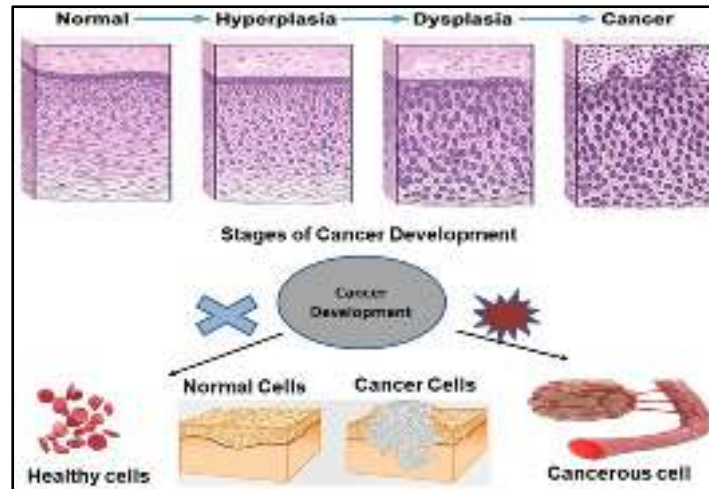


Figure 1.2 Stages of Cancer

1.3 BREAST CANCER:

Although carcinoma of the breast is a deadly foe, individuals can improve our own internal resistance against it by understanding where it originated. Healthy breasts are complicated organs composed of connective tissue, fatty tissue, milk ducts, and glands which generate milk (the lobes). Cells like this typically divide as well as regenerate themselves in a controlled way, collaborating in collectively. A change in this equilibrium indicates the beginning of cancer in the breast. These breast cell genomes are progressively modified by mutations that are either inherited or occur from external factors like toxins. The cells lack the ability to regulate their development as a consequence of these mutations, which are reminiscent of defective signals. These altered cells divide irrationally, providing abnormal clumps known as tumors, in contrast to their normally well-behaved counterparts.

Here it's crucial to remember that not every tumor is malignant. Despite becoming abnormal, benign tumors stay within the tissue and do not spread to surrounding tissues. Tumors that are malignant, nevertheless, have a risky tendency. The limits which frequently contain these cancerous growths can be collapsed, permitting them to break through normal breast tissue and possibly migrate across different organs. A characteristic that distinguishes advanced breast cancer distinctly is its ability for proliferation or metastasis. This indicates why detection at an early stage is so vital. Initial identification of the cancer provides medication yet more successful and additionally inhibits it from proliferating.

There is a diversity in the specific gene mutations which trigger breast cancer. The hormones like progesterone as well as estrogen may trigger certain types of cancer, whereas additional variables are completely unrelated. Developing recommendations about medication is made easier by understanding of the fundamental abnormalities. The occurrence of breast cancer is an ongoing procedure as opposed to one major occurrence. The initial spark can be supplied by the initial alterations but for the cancer to propagate and increase, additional genetic modifications are frequently needed. This emphasizes how essential it is to maintain a healthy lifestyle and limit our contact with carcinogens because these factors may influence the possibility that novel mutation might develop.

Researchers continually strive to enhance the early detection, treatment, and medical care of breast tumors through discovering further regarding the molecular and molecular factors that cause the cancer. Through this comprehension, we may take action towards this deadly disease while working into an era whenever breast cancer ceases to be a cause for nervousness.

1.4 DRUG REPURPOSING: A NEW HOPE IN CANCER TREATMENT:

Repurposing drugs, Drugs that are currently accessible through the market with certain goals can become unpredictable with recently discovered targets. A key that effectively unlocks a lock, for illustration, may not work with an alternate secure or could block. To be able to guarantee safety and effectiveness, researchers needed to assess these possible hyperlinks in tremendous detail. In addition, determining the appropriate quantity for an entirely novel usage takes a little longer (*Luo et al. 2020*).

Determining the best proportion for the new use is vital for drug repurposing. An additional aspect that might aggravate proceedings is prospective obstacles to law. Although an entity might have obtained a medicinal product's innovation for purposes of its own, there are also additional variables to take into consideration when repurposing the patent. Repurposing medications provides tremendous advantages notwithstanding all of these obstacles. Repurposing is a crucial tool in the battle towards cancer because it may result in the creation of therapies more quickly, fewer resources allocated to investigation that could have been used for other vital topics, and a perhaps higher chance of being successful than with completely new drugs. It's like bestowing superpowers, time, resources, and perhaps even countless lives upon prescription medicines.

1.5 REPURPOSED DRUG ZEATIN:

Plant hormones for growth termed the zeatin could be useful in the struggle with cancer. It is one of the members of the cytokinin substance family, a few of which have shown their effectiveness in the death of cancer cells. On the contrary, zeatin research is not conclusive. While certain studies indicate potential benefits additional studies have not discovered any appreciable effects. Researchers are currently looking into how cytokinins, such as zeatin, affect cancerous cells based on their structural makeup. This may open up the way towards the creation of stronger anti-cancer medications made from cytokinins. But there are still difficulties. The challenges that must be addressed are ensuring that these medications precisely target cells that are cancerous and developing

efficient delivery systems. All things considered, zeatin provides a surprising potential for future therapeutics, even though its role in treating cancer is currently being investigated.

CHAPTER 2

LITERATURE REVIEW

2.1 ROLE OF EZH2 IN OTHER CANCERS

2.1.1 Prostate Cancer:

A protein called EZH2 that has the capacity to alter histones has a dual impact on prostate cancer. EZH2 aids in the maintenance of regular growth and development in healthy cells. EZH2, however, is frequently overexpressed in prostate cancer cells. Overproduction of EZH2 has the ability to mute tumor-suppressive genes and activate those that support the growth and survival of cancer.

EZH2's role in castration resistance in prostate cancer is among its worrisome characteristics. One popular treatment is androgen deprivation therapy, which lowers testosterone levels in an attempt to starve prostate cancers. Regrettably, EZH2 overexpression is associated with the resistance that certain malignancies exhibit to this treatment. Furthermore, EZH2 has the ability to affect prostate cancer cells' capacity to change their form. These cells typically create a distinct tissue layer. These cells may undergo a metamorphosis brought on by EZH2, which would make them more invasive and migratory and raise the possibility of cancer metastasis. Moreover, EZH2 can increase the number of cancer stem cells, which are the main factor in tumor renewal. Because of its complex function in the advancement of prostate cancer, EZH2 is a prospective target for new treatments. Drugs that specifically target EZH2 are being developed by researchers, and positive outcomes from early clinical studies have been observed. To prove the long-term efficacy and safety of these medications in the fight against prostate cancer, more research is necessary.

2.1.2 Liver cancer:

One of the main adversaries in the battle against liver cancer (HCC) is EZH2. This protein prys open the door to unchecked cell development like a molecular crowbar. Genes that typically inhibit cell division are silenced by EZH2, whereas genes that encourage the

growth of HCC cells are activated concurrently. The malignant process is fueled by this unrestrained growth. The evil role that EZH2 plays doesn't end there. It can also initiate an EMT program, which is a cellular shape-shifting program. Liver cells often have a distinct structure and remain stationary. By making these cells more invasive and mobile, EZH2 raises the possibility that HCC will spread to other body organs (*Lee, JS, et al. (2018)*).

Moreover, the body's defenses against cancer are weakened by EZH2. A functioning immune system is capable of recognizing and eliminating cancer cells; however, EZH2 complicates this procedure. It inhibits immune cells that help the body fight HCC, such as T cells and natural killer (NK) cells. Research has even indicated a possible function for EZH2 in more aggressive tumors, with a correlation between elevated levels of the protein and worse prognosis for patients with HCC. Because of its complex role in the development of HCC, EZH2 is a good candidate for new treatment approaches. Drugs targeting EZH2 are being developed by researchers in an effort to stop the proliferation of cancer cells, prevent metastasis, and maybe enhance patient outcomes. To confirm these medications' long-term efficacy and safety in treating liver cancer, more research is necessary.

2.1.3 Lung Cancer:

Depending on the particular type of lung cancer, EZH2's impact is not always positive. The most common type of lung cancer, non-small cell lung cancer (NSCLC), frequently causes EZH2 to become hyperactive. Through an acceleration of cell proliferation, migration, and invasion, this hyperactivity promotes the spread of cancer. Additionally, EZH2 might play a role in the resistance of lung cancer cells to the standard chemotherapeutic medication cisplatin. However, in the case of lung adenocarcinoma, an NSCLC subtype, things get complicated. In this case, research indicates that EZH2 may function as a tumor suppressor. This indicates that the EZH2 gene has undergone mutations or deletions, and that its loss of function may be causing unchecked cell proliferation via many mechanisms. In general, patients with lung cancer have a worse prognosis when their EZH2 levels are elevated. This suggests that EZH2 activity may

serve as a marker for less likely to survive malignancies that are more aggressive. The differing impacts of EZH2 in lung cancer serve to emphasize how intricate the illness is. Taking advantage of this, scientists are looking into EZH2 as a possible target. Their goal is to create treatments that can either stop it from promoting tumors in non-small cell lung cancer (NSCLC) or bring back its possible tumor-suppressive function in lung adenocarcinomas. To identify the best treatments, however, based on the particular form of lung cancer and the underlying EZH2 activity, more investigation is required.

2.1.4 Breast Cancer:

Breast cancer is accelerated in its development and spread by EZH2, an evil force in the disease. It suppresses genes that typically limit cell division, fostering an environment that is favorable for unchecked growth—a trait that is characteristic of cancer. The evil deeds of EZH2 don't stop there. It can start a process known as epithelial-mesenchymal transition (EMT), in which breast cells that are normally stationary become mobile and invasive. This dramatically raises the risk of metastasis, or the spread of cancer to other organs. Moreover, resistance to tamoxifen, a prominent treatment for estrogen receptor-positive breast cancer, is associated with overexpression of EZH2. This resistance makes tumor growth more difficult to manage. Research has even found a correlation between higher levels of EZH2 and more aggressive types of breast cancer, indicating that this gene may serve as a predictor of worse patient outcomes. EZH2 is a prospective target for novel therapeutic approaches due to its complex role in the advancement of breast cancer. Drugs targeting EZH2 are being developed by researchers in an effort to suppress the proliferation of cancer cells, prevent metastasis, and maybe overcome resistance to treatment. To confirm these medications' long-term efficacy and safety in treating breast cancer, more research is necessary.

2.2 ROLE OF INFLAMMATORY GENES IN BREAST CANCER:

2.2.1 Nuclear factor kappa B (NF- κ B):

Normally a regulator of immunity and inflammation, a molecule called NF- κ B becomes a villain in breast cancer. Its activity goes rogue, promoting aggressive cancer behaviour. NF- κ B is usually inactive, but cellular stresses activate it. Once activated, it travels to the cell's control center (nucleus) and influences various functions, including those involved in cancer progression. One major concern is NF- κ B's role in fueling uncontrolled cell growth. It activates genes that make cancer cells multiply rapidly. Additionally, it disrupts the natural cell death process, allowing damaged cancer cells to survive and contribute to tumor growth. Furthermore, NF- κ B might influence metastasis, the spread of cancer cells. It may play a role in enabling cancer cells to detach from the primary tumor and invade healthy tissues. Due to its role in promoting cancer, researchers are exploring ways to target NF- κ B for treatment. The goal is to develop therapies that specifically target the abnormal NF- κ B activity in cancer cells without affecting healthy cells. This approach could potentially hinder tumor growth and metastasis.

2.2.2 Tumor Necrosis Factor α (TNF- α)

When it comes to breast cancer, tumor necrosis factor α (TNF- α) is a double-edged sword. It is an essential component of the immune response in healthy cells, controlling inflammatory and thwarting illness. However, TNF- α takes a negative turn in breast cancer. It can encourage the proliferation and propagation of cancerous cells and potentially increase their resistance to therapy. The increased production of TNF- α by both the malignant cells and the cells of the immune system surrounding the tumor causes an additional shock. Researchers are investigating therapeutic approaches aimed at TNF- α . The possible therapies include antibodies that inhibit its function or substances that tamper around its receptors. The difficulty, therefore, is in creating methods that neutralize TNF- α in cancer cells without impairing the body's immune system [25,26].

2.2.3 Interleukin-6 (IL-6).

A different approach signaling protein involved in breast cancer is called interleukin-6 (IL-6). In healthy cells, it serves as a messenger, but in carcinoma, it can become an antagonist. By encouraging DNA replication and averting mitochondrial death, IL-6 can promote the proliferation and existence of cancerous cells. Moreover, it initiates chronic inflammation in the tumor microenvironment by acting as a fire starter. This aggressive mix promotes the growth of cancer cells and may even have an impact on metastasis, the spread of cancer cells. Furthermore, elevated levels of IL-6 have been associated with treatment resistance, which complicates the management of the illness. Sources of IL-6, like TNF-alpha, include the tumor's surrounding immune cells, any adjacent adipose tissue, and even the cancer cells themselves. Researchers are investigating methods of targeting IL-6 for therapeutic purposes, such as antibodies that obstruct its function or compounds that tamper with its receptors on cancerous cells. The difficulty, though, is in creating treatments that specifically address IL-6's detrimental impact on cancer while preserving immune function. In the future, treating breast cancer may benefit from combining IL-6 targeting strategies with other treatments.

2.2.4 Interleukin- 1 β (IL-1 β)

Another cytokine that gets involved in breast cancer is called interleukin-1 beta (IL-1 β), and unlike TNF-alpha and IL-6, it primarily works to agitate inflammatory. It starts a metabolic inferno that can accelerate the division of cancerous cells [28]. Additionally, IL-1 β may aid cancer cells in separating from the primary tumor and invading healthy tissues, which is another way that metastasis, or the spread of cancer, is thought to occur. Even worse, it could prepare distant tissues for the entry of metastatic cancer cells by fostering an inviting atmosphere there. Furthermore, elevated levels of IL-1 β are becoming known as a possible cause for therapy tolerance. Like other types of cytokine players, the primary producers of IL-1 β within the tumor microenvironment comprise specialized lymphocytes and immune cell populations. Scholars are examining approaches to specifically target IL-1 β , such as antibodies that counteract its function or compounds that obstruct its signaling route in cancerous cells. The difficulty, though, is in

creating treatments that specifically target IL-1 β 's detrimental effects on cancer without unintentionally impairing the immune system's capacity to combat cancer cells. In the future, treating breast cancer may benefit from combining IL-1 β targeting strategies with other treatments.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

With the goal to focus on the NF κ B, TNF α , IL β , and IL 6 cascade in the development of breast cancer, particularly to look into the potential of repurposing zeatin. The use of this approach might increase the efficacy of the disease's standard therapies.

3.2 SCOPE:

The aim of this research is to investigate EZH2's potential as a breast cancer therapy. There are many advantages to repurpose current medications for novel use, especially when low-risk groups compounds are involved. This strategy is particularly useful for treatment of uncommon disorders like cancer because it can be more economical as well as quicker than conventional drug discovery techniques.

3.3 OBJECTIVES:

- IC₅₀, from concentration experiments, is the dosage causing 50% inhibition, crucial for refining therapies.
- Assess Zeatin's ability to hinder breast cancer cell growth.
- Examine Zeatin's impact on gene expression related to cancer progression.
- Investigate Zeatin's influence on hormone sensitivity in breast cancer cells.

CHAPTER 4

MATERIALS AND METHODS

4.1 *INSILICO STUDIES*

4.1.1 *InSilico* Characterization of Zeatin

a) Pharmacokinetics and Drug-likeness Predictions

Computational technologies such as SwissADME and Molsoft L.L.C. are accelerating drug discovery. These instruments aid in the prediction of small molecule behavior in the body and prospective therapeutic success. A web-based tool called SwissADME examines important characteristics such as a drug's ability to pass through fatty tissues and interact with proteins that influence absorption. Conversely, Molsoft L.L.C. concentrates on a drug's molecular properties and appropriateness for expansion, or drug-likeness. Researchers can improve drug candidates and increase the likelihood of successful clinical studies through employing this knowledge to create more potent drugs. By offering insightful information on a drug's pharmacokinetic profile and molecular characteristics, these technologies are transforming drug development and eventually resulting in more logical approaches to drug design and improved pharmacological agents.

b) Toxicological Predictions

ProTox-II network along with additional web pages are used by researchers in their hunt for safe and effective medications. This virtual laboratory examines and forecasts the possible toxicity of tiny compounds and substances. ProTox-II turns out to be a useful tool for medication makers by providing early insights into these detrimental consequences. It enables them to assess and refine drug candidates for safety and effectiveness before allocating additional funds to potentially hazardous alternatives. This guarantees that the medications in the development pipeline are just the most promising ones.

4.1.2 Protein Preparation

Investigators explored the structure of a protein known as EZH2 in order to start creating a drug. They used tools to tidy up the three-dimensional models has been taken from a protein database (PDB) so they could concentrate on the core protein. Subsequently, an additional piece of programming examined the protein's docking sites, places where medications can bind in order to decipher the arrangement of amino acids. This thorough structural characterization of the protein paves the way for the creation of new drugs.

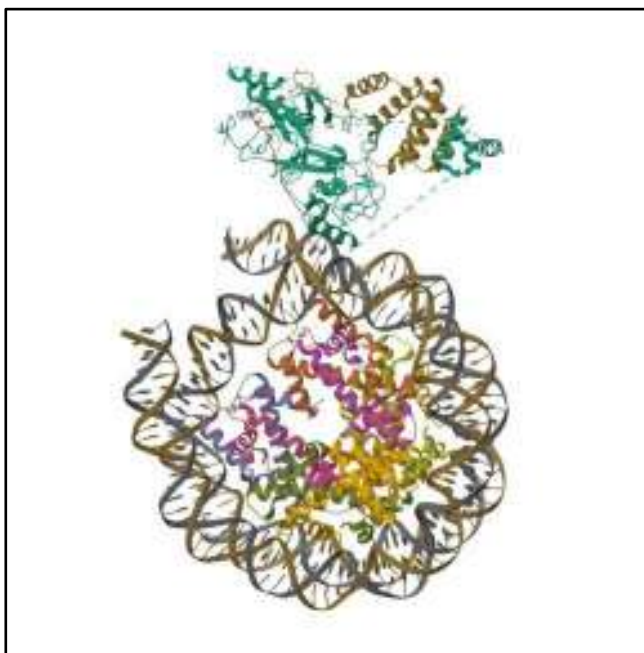


Figure 4.1 Structure of EZH2 Protein

4.1.3 Ligand Preparation

Information about the possible drug chemical in nature Zeatin was obtained by researchers. This contained structures that were taken from the PubChem database, both 2D and 3D. They also managed to obtain a SMILES string, which is a simplified molecular representation of the medication. The Open Babel online tool was used to convert the 3D structure, which was originally in the SDF format, to the more widely used PDB format. Lastly, in order to guarantee correctness, they carefully examined the 3D structure using Molegro software and eliminated any unnecessary molecules, producing an accurate depiction of Zeatin's molecular composition.

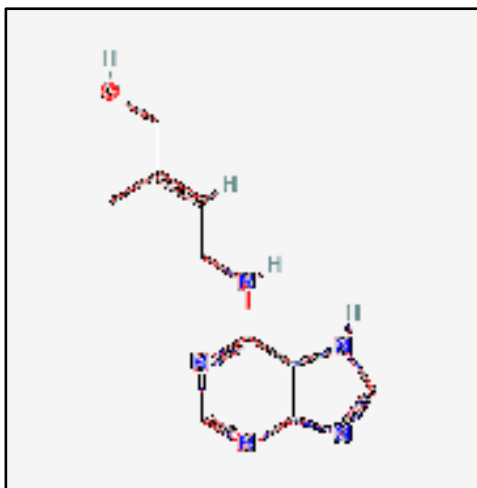


Figure 4.2 2D Structure of Zeatin



Figure 4.3 3D Structure OF Zeatin

4.1.4 Molecular Docking using PyRx Software

From exporting a protein and drug molecule to assessing the outcomes, PyRx walks researchers through every step of the process. Initially, PyRx was used to load the drug (ligand) and protein (macromolecule) independently. The next step was minimizing the energy of the ligands, which maximizes their structural efficiency for docking. The protein and ligand structures were then transformed into a format that could be used with PyRx's Vina Autodock binding algorithm. The following essential step was to define a grid box surrounding the protein's expected binding site. In essence, this box instructs the software where to look for possible interactions. PyRx ran docking experiments using the grid box setup to determine how well the medication fits into the protein. The docked poses with the highest binding scores were then determined by the program. Ultimately, the optimal docked structures were extracted for additional examination. These structures were shown in 2D and 3D using Biovia Discovery Studio, which enabled researchers to closely study the connections and interactions between the drug component and the structure of the protein.

4.1.5 Validation Docking using iGEM Software

Researchers employed iGEMDOCK, another virtual screening tool, to validate PyRx results. This easy-to-use program is intended primarily for drug development and is freely available for non-commercial research. Van der Waals forces, hydrogen bonds, and electrostatic forces are the main topics of iGEMDOCK's detailed analysis of the interactions between the drug molecule and the protein. Next, it makes predictions about how these forces interact to affect binding. Docking simulations were carried out by the researchers after importing the constructed protein and drug structures into iGEMDOCK. The subsequent binding score added to the confidence in the therapeutic molecule's potential by validating the results from PyRx. Researchers were able to increase the dependability of their drug discovery findings by utilizing both PyRx and iGEMDOCK.

4.2 INVITRO STUDIES:

4.2.1 Mammalian cell culture:

The cell lines originate from the National Centre for Cell Science (NCCS), Pune, which are known as HeLa cells - Normal Fibroblast cells and MDA MB 231. These cells were then maintained in T-25cm² flasks at 37°C and 5% CO₂ in an incubator with humidification using Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% Foetal Bovine Serum (FBS), 100 mg/L penicillin, and 100 mg/L streptomycin. Trypsinization was the method employed to subculture the cells after they acquired 80% to 90% viability. About 2 milliliters of phosphate buffer saline (PBS) were utilized for rinsing the cell monolayers. Following that, 0.5 to 2 mL was added, and the CO₂ incubator was left for one minute. After aspirating the solution, it went through a centrifuge for three minutes at 900 rpm. The pellet had been collected after which it was resuspended in 2 milliliters of 10% FBS DMEM medium. The cells were subsequently separated into four sterile, clean bottles, with each containing 0.5 mL. The bottles were subsequently stuffed to a capacity of 2 mL with DMEM medium.

TYPE OF CELL LINE	CELL LINE NAME	MEDIUM USED
Normal or Control cells	HeLa cells	DMEM with 10% FBS
Cancer cells	MDA MB 231	DMEM with 10% FBS

Table 4.1 List of Cell lines used

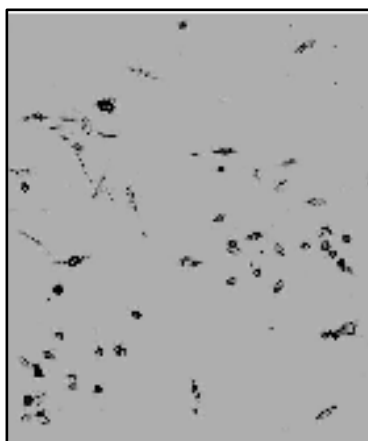


Figure 4.2.1 HeLa Cells

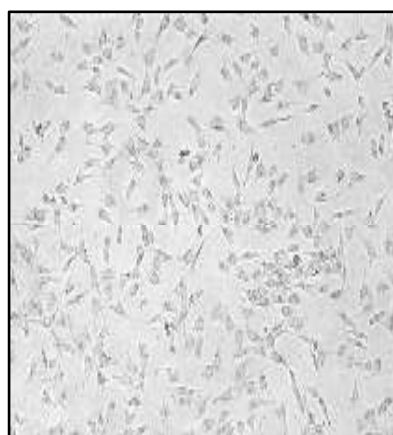


Figure 4.2.2 MDA MB 231 Cell Line

4.2.2 Preparation of drug: Zeatin;

1 mL of DMEM, 1 μ L of DMSO, and 1 milligram of Zeatin were mixed together to formulate a solution. Subsequently the suspension went through vortexes until it had entirely disintegrated. Until they were required yet again, the tubes had been kept at 4°C.



Figure 4.2.3 Dissolved Drug – Zeatin

4.2.3 MTT Cytotoxicity Assay

a) Assessment of anticancer activity of Zeatin on HeLa cell line:

A volume of 100µl was utilized to seed 96-well plates with 1×10^4 HeLa Normal cells at 90% their confluence. The cells were subsequently grown at 5% CO₂ in an incubator with humidity for the entire day. Different concentrations of dissolved drugs were administered to the cells on the subsequent day: 5 µL, 10 µL, 15 µL, 20 µL, 25 µL, and 30 µL. The cells were grown for a 24-hour period at 5 percent CO₂ in a moistened incubator. The MTT assay had been applied to the cells that were cultured. Dilute the MTT salt in PBS at a 5 mg/mL concentration to generate the MTT solution. Following a vortex of the mixture, it was finally purified. Once a component of it had been stored for future use at -20°C. The solution made from the cell cultures was thrown away in order to perform the test. 50 µL of serum-free medium and 50 µL of MTT solution was added to each well. With that, the plate was then covered in aluminum foil and left to incubate for three hours at 37°C. Discard the medium and dye after an incubation period. Hydrate each well using 150 µL of DMSO (MTT solvent). After wrapping the plate with foil, give it a 15-minute shake using an orbital shaker. In order to completely disintegrate the MTT formazan, the solution was pipetted on multiple occasions. At OD = 570 nm, the absorbance was subsequently detected.

b) Assessment of anticancer activity of Zeatin on MDA MB 231 cell line:

100µl containing 1×10^4 MDA MB 231 cells in them A plate with 96 wells had been seeded at 90% confluence, and the cells were grown for 24 hours at 5% CO₂ in a moistened incubator. The drugs that had been neutralized were added to the cells on the subsequent day at the respective concentrations: 5 µL, 10 µL, 15 µL, 20 µL, 25 µL, and 30 µL. For a full 24 hours, the cells were nurtured in an incubator humidified with 5% CO₂. Now the cells that were grown were exposed to the MTT test. Dilute the MTT salt in PBS at a 5 mg/mL concentration to generate the MTT solution. Upon vortexing the mixture, it was screened. Following that, it had been stored for later use at -20°C. The solution made from the cell cultures was flung away so as to carry out the test. 50 µL of serum-free medium and 50 µL of MTT solution have been added to each well. From that, the plate had been wrapped in aluminum foil and placed in the incubator for three hours at 37°C. Discard the medium and dye after the incubation phase. Fill each of the wells with 150 µL of DMSO (MTT solvent). While wrapping the plate with foil, provide it a 15-minute shake utilizing an orbital shaker. For complete dissolution of the MTT formazan, the mixture was pipetted many times. At OD = 570 nm, the wavelength of absorption was finally detected.

Percentage of Inhibition = $100 - [((\text{Control OD} - \text{Treated OD}) / \text{Control OD}) * 100]$



Figure 4.2.4 MTT Assay for Normal Cells (HeLa)



Figure 4.2.5 MTT Assay for Cancer Cells (MDA MB 231)

4.2.4 Dual Acridine Orange/Ethidium Bromide (AO/EB) Fluorescent Staining

It has been used for examining the modifications in membranes of cells which take place during the process of apoptosis. Although Ethidium Bromide (EB) exclusively stains cells that are damaged, Acridine Orange (AO) is a crucial dye that can stain cells that are alive and dead. Green staining will be administered to the living cells or the cells that are currently in the initial phases of apoptosis. Orange staining occurs due to late apoptotic cells consuming EB dye. A 6-well plate holding 1×10^4 MDA MB 231 cells had been seeded with 3 mL of DMEM media, and the cells were cultivated for 24 hours at 5% CO₂ in a moistened incubator. The cytotoxicity of the drug was made in a development environment and added to the cells that were developing at an IC₅₀ concentration on the next day. As a control, three of the wells have been kept. After that, these cells were developed in an incubator with humidity at 5% CO₂ for an entire day. Ten milligrams per milliliter of PBS solution was employed to generate the stock solutions for AO and EB. The tested solution was made by diluting 1 μ L of stock solution with 100 μ L of PBS, which led to a final level of concentration of 1 mg/mL for both AO and EB. The two stains are mixed in a 1:1 ratio to generate the AO/EB stain. Throughout the period of incubation, 20 minutes were expended in each well holding 10 μ L of each stain (only AO in Control 1 and Treated 1, only EB in Control 2 and Treated 2, AO/EB in Control 3 and Treated 3). Following that, an inverted fluorescent microscope was employed to view the cells.

4.2.5 Total RNA Isolation

The cytotoxicity treatment was administered at an IC50 concentration to the cancer cells. For an entire day, it was kept in an incubator filled with 5% CO₂. The following day, an ice-cold PBS solution was employed for washing the cells. 1 mL of Trizol is added to the suspended cells, then scratched. 250 µL of chloroform was added to the trizol cell lysate, combined, and permitted to settle at room temperature for a period of five minutes. Following that, the entire mixture was centrifuged for five minutes at 10,000 RPM. From a component of the aqueous phase was pipetted out through an entirely new 1.5 mL tube without disrupting the contents, and it was stored on ice. Before that, the aqueous phase was mixed with 550 µL of isopropanol and centrifuged for 20 minutes at 4°C at 10,000 rpm. After retrieving it, the pellet was rinsed two times using 75% ethanol and sterile, nuclease-free water. The pellet was subsequently air-dried and resuspended in 25 µL of sterile water that did not contain nuclease.

From that, the RNA was diluted 1:40 with sterile water free of nuclease. The reader of a microplate was employed for reading the solution at 260 and 280 nm with the objective to determine the A₂₆₀:A₂₈₀ proportion and quantify the total amount of RNA per µL. Once that, the RNA was stored at -20°C until necessary.

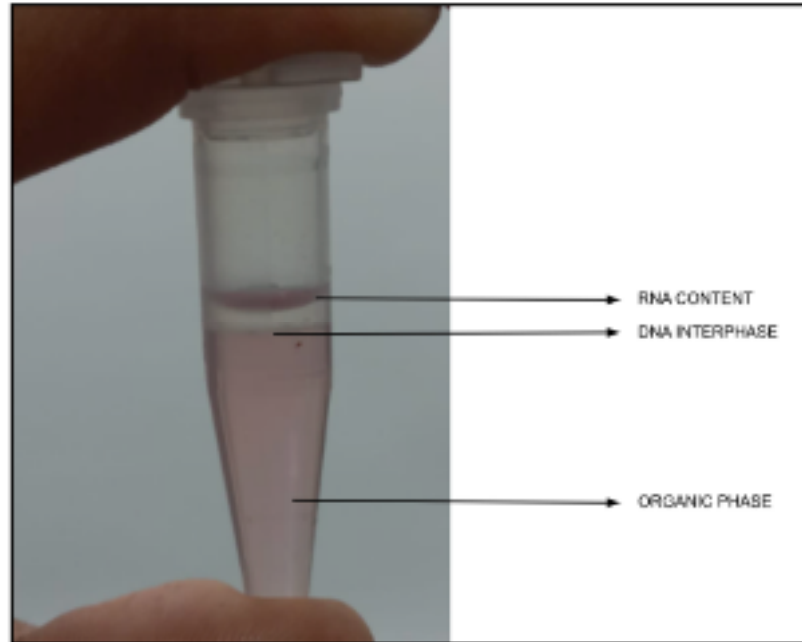


Figure 4.2.6 Phase Separation during RNA Isolation

4.2.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis has been employed for evaluating RNA integrity. Initially agarose was dissolved in 20 milliliters of 1x Tris/Boric acid/EDTA (TBE) buffer and warmed until it reached breakdown in order to create a 1% agarose gel. By stirring in ethidium bromide (EtBr), the resultant mixture was transferred onto a gel tray and a comb was inserted inside. During a 15 to 30 minute solidification period, the gel in the tray was immersed in a 250 mL gel tank containing 1x TBE buffer. Following that, 10 μ L of total RNA and 6 μ L of 6x gel loading dye were mixed together, deposited onto the gel, and electrophoresed for 30 minutes at 100 V. According to UV absorbance, gel visualization and documentation were performed using the Gel Doc XR+ Gel Documentation System (Biorad, USA). This method made it easier to determine the reliability of RNA, which is crucial for numerous purposes in molecular biology.

4.2.7 cDNA Conversion

Using the iScript™ cDNA Synthesis Kit, 100 ng of total RNA has been converted to cDNA in two phases as per the manufacturer's instructions. Using the technique, 15 µL of total RNA, 4 µL of the master mix, and 1 µL of the RNA reverse transcriptase (RT) enzyme were mixed together to generate a 20 µL quantity. In a temperature cycler, the tubes were initially primed for five minutes at 25°C, followed by activation for forty-six minutes at 46°C, and then inactivated for one minute at 95°C, after being put away at 4°C. To be utilized afterwards, the tubes were maintained into preservation at -20°C.

4.2.8 Designing of Real-Time quantitative Polymerase Chain Reaction Primers

Utilizing the Eurofins genomics primer developing program, the primers for the target genes (NF-B, IL 6, TNF, and IL 1) were generated and Barcode Biosciences, Bangalore, was given the task of manufacturing them. Several criteria were chosen as the best choices for this primer: Melting temperature (T_m): 65 to 72 oC; annealing temperature (T_a): 50 to 57 oC; primer length: 18 to 20 bp; GC content: 40% to 55%. For the purpose of being utilized later, the primers were reduced to a working concentration of 1 µM after being dispersed in sterile, nuclease-free water.

S.NO	TARGET GENE	GENE ID	FORWARD PRIMER	REVERSE PRIMER	AMPLICON LENGTH (bp)	T _a (°C)
1	NF-κB	4791	CGAATGGACAAG ACAGCAG	CCGGAACACAA TGGCATAC	177	53.7
2	IL 6	3569	ATAACCACCCCT GACCCCAAC	ATACCTCAAAC TCCAAAAGACC	149	55.1
3	TNF α	7124	AACCATTCTCCTT CTCCCC	TTTCACCTCCAT CCATCCTC	194	54.3
4	IL 1β	3553	CTCTCACCTCTC CTACTCAC	ACACTGCTACT TCTTGCCC	196	51.5

Table 4.2 List of Primers used for qRT-PCR analysis



Figure 4.2.7 Forward and Reverse Primers

4.2.9 Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)

SensiFAST™ SYBR® Lo-ROX Kit (Bioline, UK) was employed to perform qRT-PCR as triplicate using gene-specific primers for four genes (NF- κ B, IL 6, TNF α , and IL 1 β) in accordance with the directions provided by the manufacturer. 5 μ L of SYBR Green master mix, 1 μ L each of the forward and reverse primers (3 μ M), and 3 μ L of cDNA template (50 ng) had been added. Nuclease-free water was subsequently incorporated to bring the volume to 10 μ L. The Mastercycler Nexus Gradient Thermal Cycler (Eppendorf, Germany) was employed for amplifying PCR. 95°C for 2 minutes (polymerase activation), 95°C for 5 seconds, 60 to 65°C for 10 seconds, and 72°C for 15 seconds constituted the thermal cycling conditions. It took place for 40 cycles total. The program had been set up to determine the Cycle Threshold Value (Ct value) over a 40-cycle period. The Relative Quantification ($2^{-\Delta\Delta C_t}$) approach was used to quantify the differences in expression between the target and control. For the reference collection in this approach, the ratio of gene expression is taken to be one. It is predicated on an amplification of PCR that is homogeneous across every specimen and is completely effective. Expression fold change as a proportion of the control was used to report the outcomes. Fold change in

the gene expression was interpreted as overexpression when it was >1.0 and downregulation when it was <1.0 .

$$\text{Fold Change or Relative Quantification (RQ) value} = 2^{-\Delta\Delta C_t}$$

4.3 Statistical Analysis

A strong statistical analysis and graphical tool that is frequently utilized in scientific research is called GraphPad Prism. Its simple to operate interface enables the analysis of statistics, fitting curves and data presentation easier. Prism is a popular instrument among investigators due to its ability to expedite data analysis with features like computerized error bar advancement and nonlinear regression modeling. The table below includes information about the experiments that were run.

4.3.2 Statistical Test of MTT Assay:

a) Unpaired T- Test:

The P value is reported as 0.0002, which indicates statistical significance. A smaller P value suggests stronger evidence against the null hypothesis. The three asterisks (***) next to the P value signify that the result is highly significant. The test result confirms that there is a significant difference (with a P value less than 0.05) between the groups being compared.

Unpaired t test	
P value	0.0002
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.197, df=30

Figure 4.2.8 Unpaired T Test for MTT Assay

4.3.2 AO/EB Staining

b) Chi- Square Test

This table shows the p-value and statistical significance (Chi- square test): The type of statistical test performed is Fisher's exact test. This test is used to assess the statistical significance of relationships between categorical variables in small datasets. The significant p-value indicates the significant difference in cell viability between the control group and zeatin. The p-value is less than 0.0001, which is typically considered statistically significant. Statistically significant ($P < 0.05$): Yes.

P value and statistical significance	
Test	Fisher's exact test
P value	<0.0001
P value summary	*****
One- or two-sided	NA
Statistically significant ($P < 0.05$)?	Yes

Figure 4.2.9 Chi – square test for AO/EB Staining

4.3.3 qRT-PCR

c) Unpaired T- Test

The P value is reported as 0.0158, which indicates statistical significance. A smaller P value suggests stronger evidence against the null hypothesis. The three asterisks (*) next to the P value signify that the result is significant. The test result confirms that there is a significant difference (with a P value less than 0.05) between the groups being compared.

7	Unpaired t test	
8	P value	0.0158
9	P value summary	*
10	Significantly different ($P < 0.05$)?	Yes
11	One- or two-tailed P value?	Two-tailed
12	t, df	t=3.332, df=6

Figure 4.2.10 Unpaired T Test for qRT PCR

S.NO	EXPERIMENT	STATISTICAL TEST	PURPOSE
1.	MTT Assay	Unpaired T-Test	Examine how the medication affects cancerous and healthy cells.
2.	AO/EB Staining	Chi-square Test	To determine whether the medication therapy significantly affects the cancer cells' propensity to undergo apoptosis.
3.	qRT-PCR	Unpaired T Test	To successfully analyze the data by comparing the levels of gene expression in cancer cells treated with and not treated with drugs.

Table 4.3 List of tests used for Statistical Analysis

CHAPTER 5

RESULTS AND DISCUSSION

5.1 *INSILICO STUDIES*

5.1.1 *InSilico* Characterization of Zeatin

a) General Characteristics

Pubchem ID	Smiles ID	Molecular formula	Molecular Weight (g/mol)
447123433	<chem>CC(=CCNC1=NC=NC2=C1NC=N2)CO</chem>	C ₁₀ H ₁₃ N ₅ O	219.24 g/mol

Table 5.1 General Characteristic of Zeatin

b) Lipophilicity

iLOGP	XLOGP3	WLOGP	MLOGP	SILICOS-IT	Consensus Log $P_{a/w}$
1.04	0.67	0.51	-0.03	1.02	0.64

Table 5.2 Lipophilicity of Zeatin

c) Water solubility

ESOL			
LOG S	SOLUBILITY		CLASS
	mg/mL	Mol/L	
-1.77	3.69e*00	1.68e-02	Very Soluble

ALI			
LOG S	SOLUBILITY		CLASS
	mg/mL	Mol/L	
-2.07	1.88e+00	8.56e-03	Soluble

SILICOS-IT			
LOG S	SOLUBILITY		CLASS
	mg/mL	Mol/L	
-2.94	2.53e01	1.16e-03	Soluble

Table 5.3 Water Solubility of Zeatin

d) Pharmacokinetic Parameters

GI absorption	High
BBB permanent	No
P-gp substrate	No
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
Log K_p (skin permeation)	-7.16 cm/s

Table 5.4 Pharmacokinetics of Zeatin

e) Drug-likeness

Lipinski	Yes; 0 violation
Ghose	Yes
Veber	Yes
Egan	Yes
Muegge	Yes
Bioavailability Score	0.55

Table 5.5 Drug-likeness of Zeatin

f) Medicinal Chemistry Properties

Pains	0 alert
Brenk	1 alert: isolated alkene
Lead Likeness	No; violation: MW<250
Synthetic accessibility	2.54

Table 5.6 Medicinal Chemistry properties of Zeatin

5.1.2 Molecular Docking using PyRx and Validation by iGEMDock

a) Molecular Docking using PyRx:

- Binding Affinity: -4.7 kcal/mol
- rmsd/ub: 0
- rmsd/lb: 0

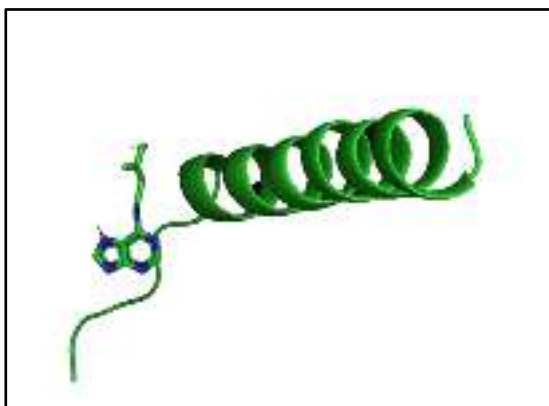


Figure 5.1.1 3D Structure of docked Zeatin and EZH2

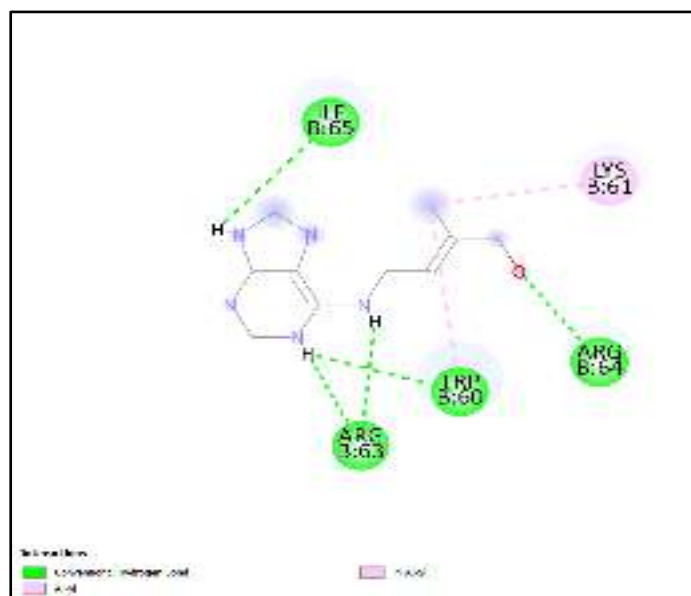


Figure 5.1.2 2D Structure of docked Zeatin and EZH2

b) Validation docking using iGEMDock

→ Energy: -77.15 kcal/mol

→ VDW: -59.65

→ HBond: -17.5

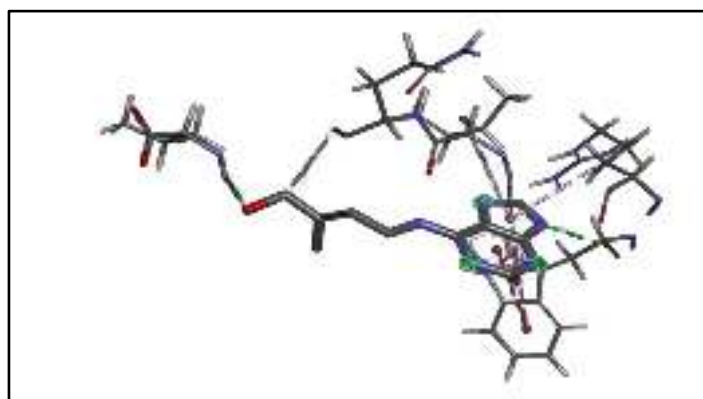


Figure 5.1.3 3D Structure of validated Zeatin and EZH2

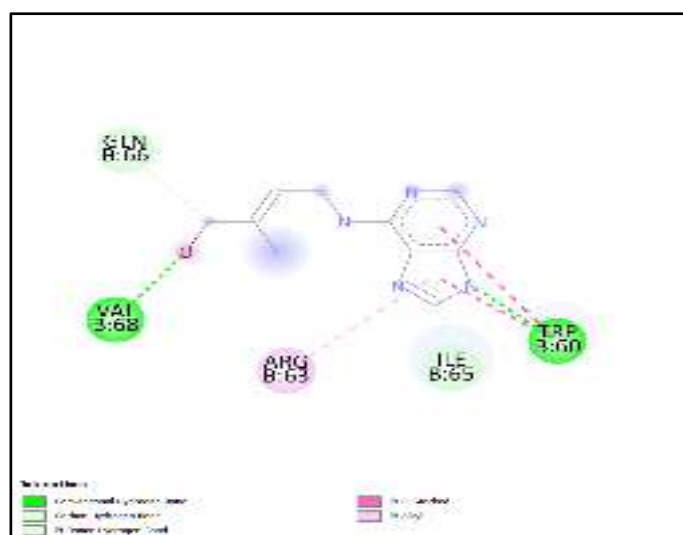


Figure 5.1.4 2D Structure of validated Zeatin and EZH2

5.2 INVITRO STUDIES

5.2.1 Anticancer activity of Zeatin on HeLa cell line:

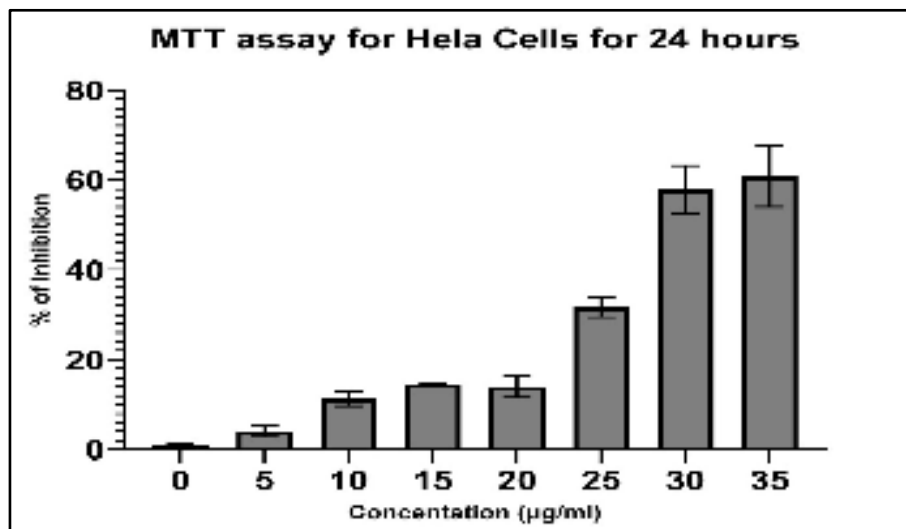


Figure 5.2.1 Anti-Cancer activity of Zeatin on HeLa cell line

5.2.2 Assessment of anticancer activity of Zeatin on MDA MB 231 cell line:

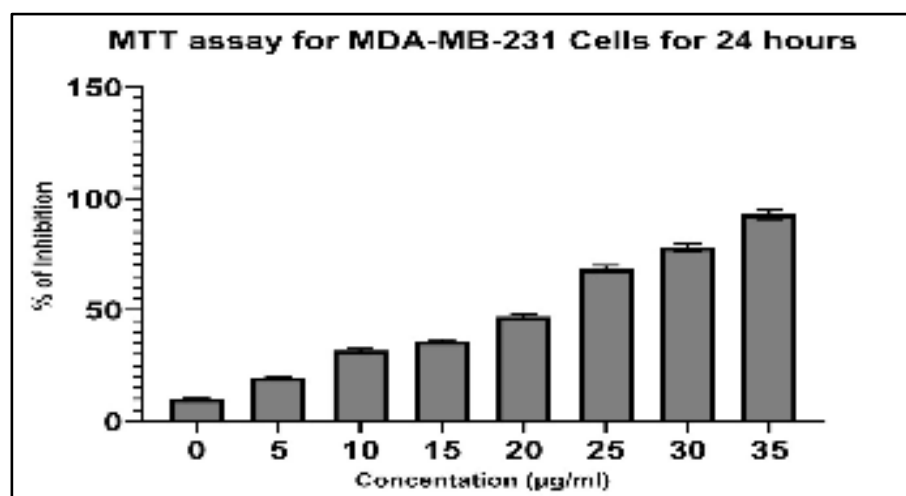


Figure 5.2.2 Anti-cancer activity of Zeatin on MDA MB 231 cell line

5.2.3 Inhibition of HeLa and MDA MD 231 cell line under the treatment of Zeatin:

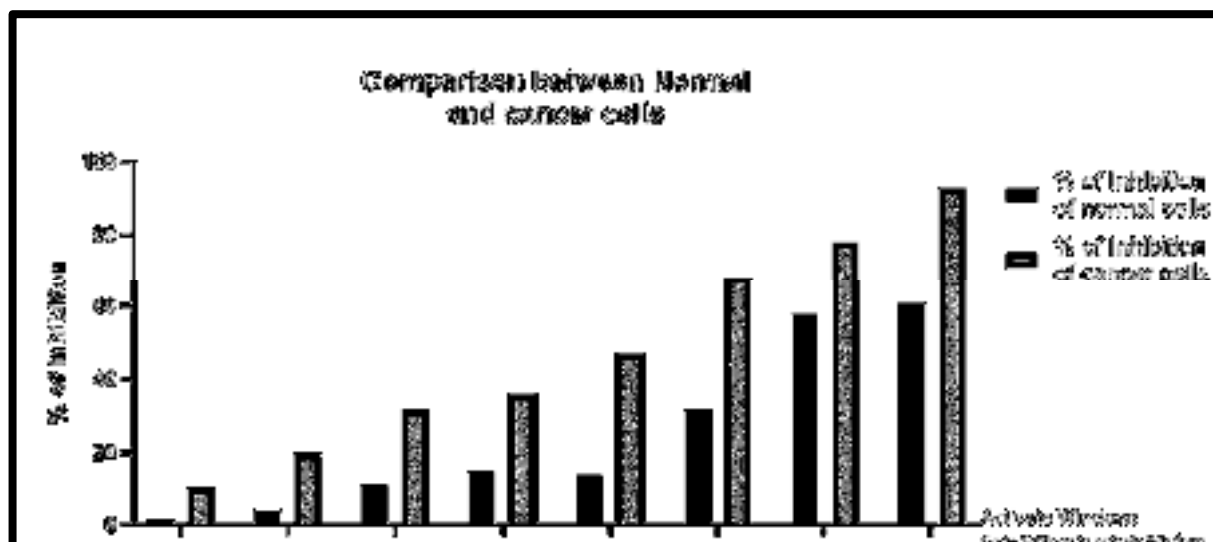


Figure 5.2.3 Inhibition Comparison between HeLa and MDA MD 231 cell line

5.2.4 Dual Acridine Orange/Ethidium Bromide (AO/EB) Fluorescent Staining

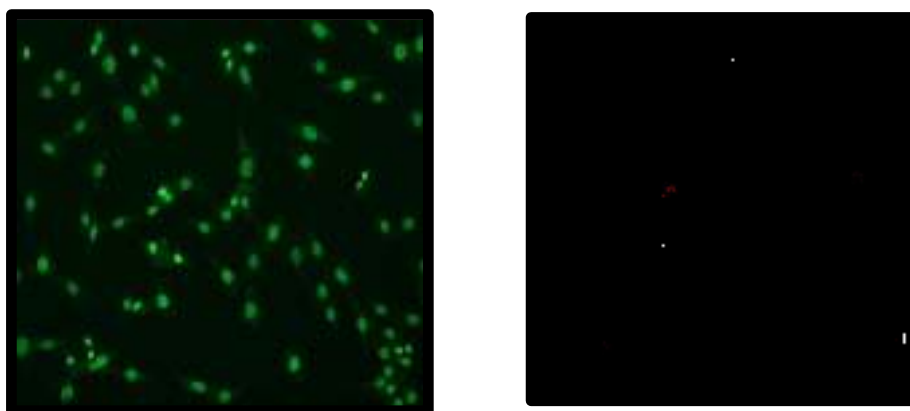


Figure 5.2.4 Control (Acridine Orange); Control (Ethidium Bromide)

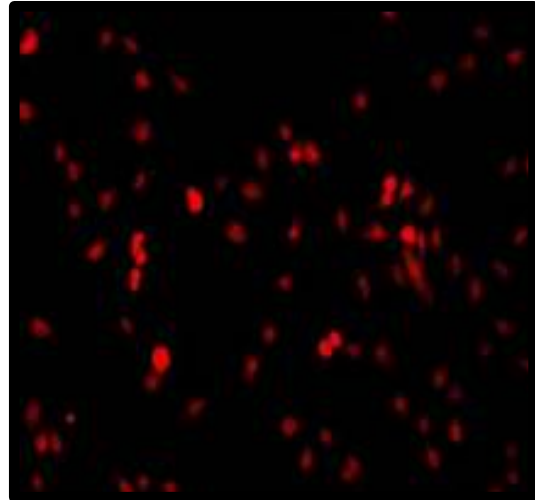
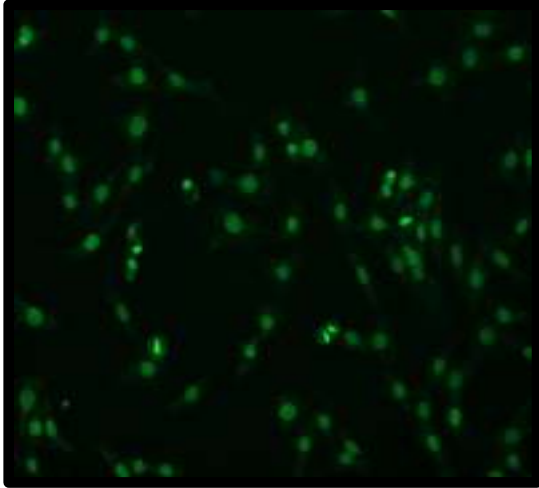


Figure 5.2.5 Treated (Acridine Orange); Treated (Ethidium Bromide)

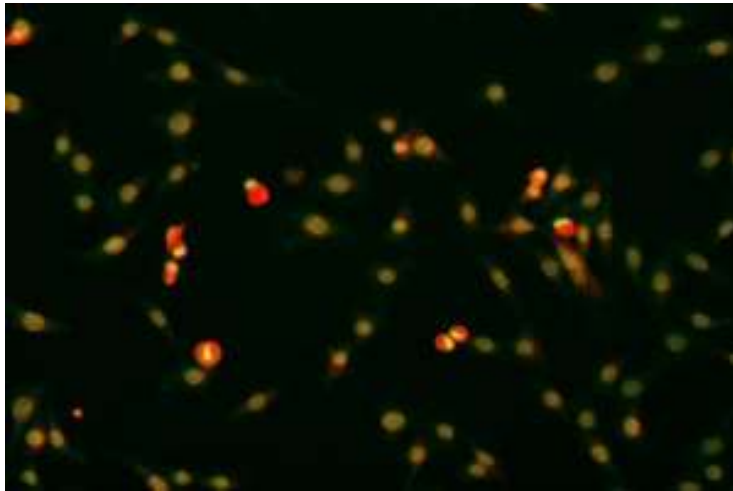


Figure 5.2.6 Treated (Acridine Orange + Ethidium Bromide)

Stains	Control	Treated
AO	274	101
EB	8	216
AO / EB	134	204

Table 5.7 Cell count after treated with Zeatin and Stains

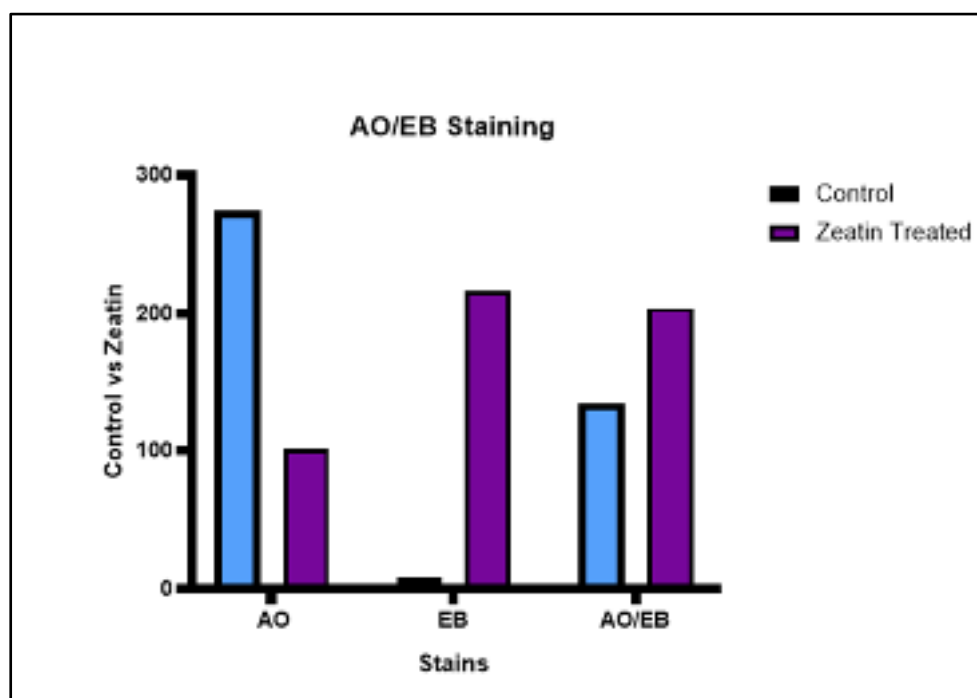


Figure 5.2.7 Graphical Representation of AO/EB Staining results

5.2.5 RNA Isolation

a) Agarose Gel Electrophoresis

After RNA isolation, agarose gel electrophoresis serves as a quality control for the extracted RNA. The best result is to see two different bands; the ribosomal RNA (rRNA) molecules, namely the 28S and 18S types, are the stars of the show. The bands are observed, it means that the RNA molecules haven't broken down. Bands indicate a RNA concentration. The intensity of the rRNA bands can also provide an indication of the amount of RNA. Agarose gel electrophoresis occasionally reveals undesirable guests genomic DNA contamination although this is less common. A large molecular weight band is visible above the rRNA superstars. This band shows up, the RNA needs to be further purified in order to get rid of the DNA before being used in other applications. Overall, agarose gel electrophoresis provides a picture of the RNA's health, ensuring it's intact and ready for further research.



Figure 5.2.8 Agarose Gel Electrophoresis

b) Quantification of RNA

After extracting RNA from drug treated HeLa and MDA-MB-231 cells using precipitation and isopropanol centrifugation, the isolated RNA appeared as clear white pellets. To ensure the RNA remained intact, these pellets were prepared for agarose gel electrophoresis and then air-dried at room temperature. The RNA samples were loaded into two wells of the gel, and after running the gel, the resulting bands were visualized using UV light with the Gel Doc XR+ Gel Documentation System (BioRad, USA). The presence of two distinct band patterns confirmed the integrity of the RNA samples. The smaller band likely corresponds to ribosomal RNA (rRNA), while the larger band likely represents messenger RNA (mRNA).

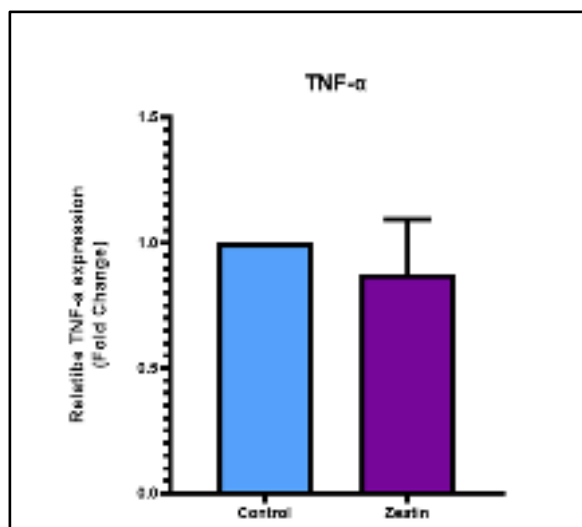
5.2.6 Synthesis of cDNA

Verifying the proper production of cDNA is a crucial stage in numerous molecular biology investigations. To do this, there are several methods. One method is to use gel electrophoresis to examine the cDNA molecules' sizes. cDNA ought to be bigger because it is a DNA replica of the original RNA template. A PCR process utilizing the cDNA is one more technique. The presence of the target gene will be indicated by the specific primers amplifying its sequence from the cDNA if the cDNA was generated correctly. Lastly, the synthesis of the cDNA can be indirectly verified by the successful completion of downstream applications that make use of it, such as gene cloning or sequencing.

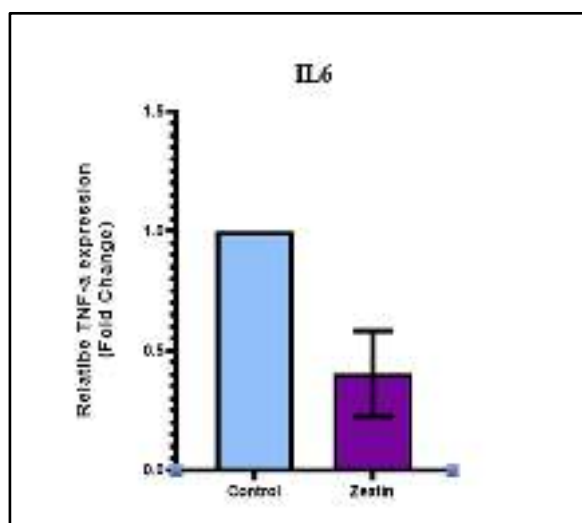
5.2.7 Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

S.No	Sample	Relative expression (Fold Change)
1	Control	1
2	TNF- α	0.875
3	IL-6	0.403388
4	IL- β	0.026465
5	NF- κ B	0.361036

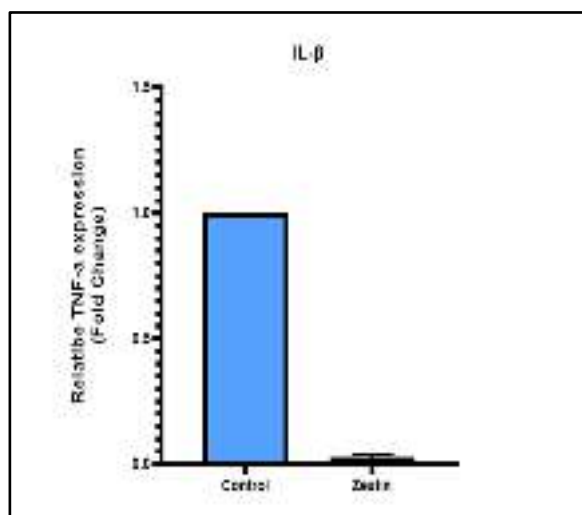
Table 5.8 Fold Change for various genes (TNF α , IL 6, IL 1 β , NF κ B)



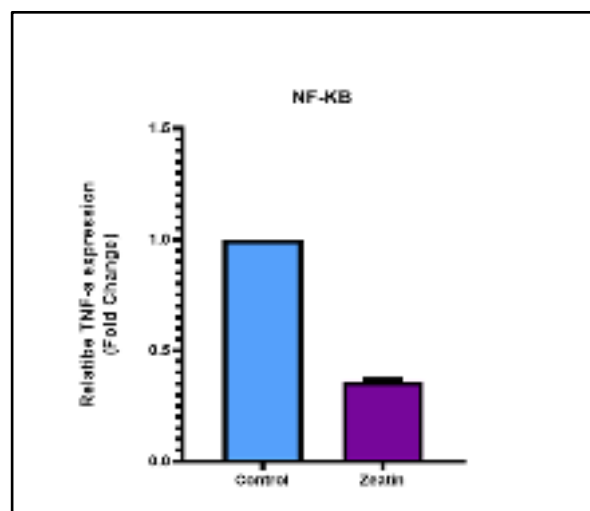
Expression of TNF α



Expression of IL 6



Expression of IL 1 β



Expression of NF κ B

Figure 5.2.9 Graphical Representation of gene regulation from RT-PCR studies

Zeatin is a plant hormone that could potentially be utilized in treating breast cancer by preventing the growth of breast cells, although more research is required to confirm this (Garmanchuk et al., 2023). It's interesting that the study focuses on the EZH2 protein as a possible target for zeatin's antiproliferative action. It is crucial to investigate the downstream effects of EZH2 inhibition, such as apoptosis or cell cycle arrest, in order to fully comprehend the mechanism. Additionally, the expression study component creates stimulating debate topics (Voller et al., 2010). Determining the precise gene expression alterations brought about by zeatin treatment may disclose other pathways that it may affect. This information may open the door to investigating possible synergy with current treatments, wherein zeatin may improve the efficacy of other medications (Yang et al., 2022). Research must continue to focus on important facets of therapeutic development. The right zeatin dosage and delivery strategy must be determined. Investigating nanoparticle technologies may be necessary to minimize damage to healthy tissues while guaranteeing efficient delivery to cancer cells. Furthermore, animal studies are required to evaluate the safety and effectiveness of zeatin in a whole organism in order to translate the existing in vitro findings into a therapeutic setting. In the end, positive results from research on animals may open the door to clinical trials and make zeatin more accessible to people with breast cancer (Wu et al., 2018).

CHAPTER 6

SUMMARY AND CONCLUSIONS

Zeatin is a plant hormone that researchers are investigating as a possible weapon in the fight against breast cancer. The ability of zeatin to stop cancer cells from growing out of control is the main topic of this investigation. The study focuses on a protein known as EZH2 and explores if zeatin targets it to prevent cell division. Comprehending this interplay may unveil the mechanism by which zeatin combats cancer. The study also examines the impact of zeatin therapy on gene expression in cancer cells, offering insights into potential wider pathways. But important questions still need to be answered. Is zeatin exclusively dependent on EZH2, or are there more processes involved? Does it selectively target cancer cells? To verify zeatin's safety and efficacy as a medication, more investigation is required, including clinical trials and investigations on animals. For zeatin to be a viable plant-based alternative to existing treatments for breast cancer, its entire mechanism of action needs to be understood.

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ANNEXURE

Submission and acceptance of abstract titled “Connecting the Dots: Breaking Barriers in Breast Cancer - A Network Pharmacology Quest into Drug Repurposing” to Monash University, Malaysia.

 MONASH UNIVERSITY MALAYSIA Uniting Minds in Global Health Sciences Monash Virtual Conference 2023   Jeffrey Cheah School of Medicine and Health Sciences	(P025) Connecting The Dots: Breaking Barriers in Breast Cancer: A Network Pharmacology Quest into Drug Repurposing Hanitha Sivakumar Sathyabama Institute of Science and Technology Breast cancer, arising from mammary gland tissues, specifically the inner linings of milk ducts and lobules, presents a formidable global health challenge, accounting for approximately 25% of annual cancer diagnoses in women. Its challenging nature lies in its tendency to metastasize to distant organs such as the liver, brain, lung, and bone, underscoring the pivotal importance of early detection for a positive prognosis and heightened survival rates. The advent of drug repurposing responds to the escalating demand for diverse, expeditious, and cost-effective drug development strategies. This approach involves reimagining the applications of approved or abandoned compounds, presenting an avenue for innovative therapeutic options and sidestepping the intricacies linked to de novo drug creation. Network pharmacology assumes a crucial role in appraising multirug interactions, furnishing a framework for comprehending the effects of administering multiple drugs against breast cancer. This method takes into account both the drugs mechanism of action and the molecular network of diseases such as cancer.
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GATE EXAM SCORE CARD

Name

HARITHA SIVAKUMAR

Registration Number

BT24S57402516

Gender

Female

Parent's/Guardian's name

SIVAKUMAR B

Date of Birth (YYYY-MM-DD)

2003-03-13

Examination Paper

Biotechnology (BT)



Photograph

Haritha.S

Signature

Marks out of 100[#]

23.58

Qualifying Marks^{##}

38.9

General

35

OBC-NCL/EWS

25.9

SC/ST/PwD