

REVITALISING NYSTATIN FOR TARGETING CELL PROLIFERATION AND GENE EXPRESSION IN BREAST CANCER STUDIES

PROJECT WORK

*Submitted in partial fulfilment of the requirements for the award of Bachelor of
Technology in Biotechnology*

By

FATHIMA SUMAIRA S (40230010)



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

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A++" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHISALAI,

CHENNAI - 600119

APRIL - 2024



Accredited "A++" Grade by NAAC | 12B Status by UGC | Approved by AICTE
www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **FATHIMA SUMAIRA S (40230010)** who carried out Project Work entitled **“Revitalising Nystatin for targeting cell proliferation and gene expression in Breast cancer studies”** under our supervision from October 2023 to March 2024.

INTERNAL GUIDE

Dr. V. Ramesh Kumar

EXTERNAL GUIDE

Dr. M. Bavanilatha

HEAD OF THE DEPARTMENT

Dr. V. RAMESH KUMAR

Submitted for Viva-voce Examination held on 05th April 2024

Internal Examiner

(Dr. S. Usha Wandhini)

External Examiner

(Dr. B. DEVI)

DECLARATION

I, **Fathima Sumaira S (40230010)**, hereby declare that the Project Report entitled **“Revitalising Nystatin for targeting cell proliferation and gene expression in Breast cancer studies”** done by me under the guidance of **Dr. V. Ramesh Kumar (Internal)** and **Dr. M. Bavanilatha (External)** at the Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of **Bachelor of Technology Degree in Biotechnology**.

DATE: 05th April 2024

PLACE: Chennai

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the **Board of Management of Sathyabama Institute of Science and Technology**, Chennai, for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. V. Ramesh Kumar, Ph.D.**, Head of the Department of Biotechnology and my Internal Project Guide for providing me necessary support and details at the right time during the progressive reviews. I would also like to express my sincere and deep sense of gratitude for his constant support and encouragement during the advancement of my project work.

I am extremely grateful to **Dr. M. Bavanilatha , Ph.D.**, for her valuable guidance, suggestions, and constant encouragement paved the way for the successful completion of my project work.

I am sincerely thankful to **Dr. A. Madan Kumar Ph.D. (Research)**, and **Dr. R.P. Oviya, Ph.D.**, for their constant support and guidance during the tenure of the project, setting the path for my project to be completed successfully.

I wish to express my thanks to **all Teaching and Non-teaching staff members of the Department of Biotechnology and International Research Centre (IRC)** who were helpful in many ways for the completion of the project.

ABSTRACT

Breast cancer, a prevalent and complex disease, poses significant challenges in healthcare globally. Despite advancements in treatment, including surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy, challenges such as treatment resistance, disease relapse, and adverse effects persist. Triple-negative breast cancer (TNBC), characterised by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression, presents unique therapeutic hurdles due to limited targeted treatment options. The overexpression of epidermal growth factor receptor (EGFR) in TNBC underscores its significance as a potential therapeutic target, yet exploitation of this target faces obstacles, including the development of resistance mechanisms. Drug repurposing emerges as a promising strategy, leveraging existing drugs for new therapeutic indications. Nystatin, an FDA-approved antifungal drug, shows potential as a repurposed agent in cancer therapy, with emerging evidence of its anticancer properties and ability to modulate EGFR signalling and inflammatory pathways in TNBC. By integrating bioinformatics approaches and gene expression studies, this thesis aims to elucidate the molecular mechanisms underlying Nystatin's anticancer effects, contributing to the development of innovative therapeutic interventions for breast cancer.

KEYWORDS: *Breast cancer, Repurposing, Nystatin, EGFR.*

TABLE OF CONTENTS

S.NO	TITLE	PAGE NO
	Abstract	i
	List of Abbreviations	vi
	List of Figures	vii
	List of Tables	viii
1	Introduction	1
1.1	Breast Cancer	1
1.2	Treatments For Breast Cancer	2
1.3	Triple Negative Breast Cancer (TNBC)	2
1.4	Epidermal Growth Factor Receptor (EGFR)	3
1.5	Repurposing Of Drugs	3
2	Literature Survey	5
2.1	Breast cancer	5
2.2	Complications in Breast Cancer Treatment	7
2.3	Role of Epidermal Growth Factor Receptor (EGFR) in Breast cancer	9
2.4	Role of various cytokines in Breast cancer progression	10
2.4.1	Nuclear Factor - kB (NF-kB)	11
2.4.2	Interleukin 1B (IL 1B)	12
2.4.3	Interleukin 6 (IL 6)	12
2.4.4	Tumour Necrosis Factor a (TNF a)	13

2.4.5	BCL 2 Family	14
2.5	Nystatin as a Drug	14
2.6	Nystatin as an Inhibitor of Lipid raft-caveolae Endocytosis pathway	15
2.7	Repurposing of Drugs in Cancer	16
3	Aim and Scope	18
4	Materials and Methods	19
4.1	<i>In Silico</i> Studies	19
4.1.1	Pan-cancer survival analysis of EGFR using TCGA	19
4.1.2	Pan-cancer survival analysis of EGFR using KM	19
4.1.3	Validation of EGFR survival analysis study in pan-cancer	20
4.1.4	<i>In Silico</i> Characterization of Nystatin	20
4.1.5	Protein Preparation	21
4.1.6	Ligand Preparation	21
4.1.7	Molecular Docking using PyRx Software	22
4.1.8	Validation Docking using iGEM Software	23
4.2	<i>In Vitro</i> Studies	24
4.2.1	Mammalian cell culture	24
4.2.2	Preparation of Nystatin	25
4.2.3	MTT Cytotoxicity Assay	25
4.2.4	Dual Acridine Orange/Ethidium Bromide (AO/EB) Fluorescent Staining	27

4.2.5	DPPH Assay	28
4.2.6	Total RNA Isolation	28
4.2.7	Agarose Gel Electrophoresis	29
4.2.8	cDNA Conversion	29
4.2.9	Designing of Real-Time Quantitative Polymerase Chain Reaction	30
4.2.10	Quantitative Real-Time Polymerase Chain Reaction	31
4.3	Statistical Analysis	32
5	Results and Discussion	34
5.1	<i>In Silico</i> Studies	34
5.1.1	Pan-cancer survival analysis of EGFR using TCGA	34
5.1.2	Pan-cancer survival analysis of EGFR using KM	35
5.1.3	Validation of EGFR Survival Analysis Study using GEPIA	36
5.1.4	<i>In Silico</i> Characterization of Nystatin	37
5.1.5	Molecular Docking using PyRx and Validation by iGEMDock	45
5.2	<i>In Vitro</i> Studies	46
5.2.1	MTT Cytotoxicity Assay	46
5.2.2	Dual Acridine Orange/Ethidium Bromide (AO/EB) Fluorescent Staining	45
5.2.3	DPPH Assay	48
5.2.4	RNA Isolation and cDNA conversion	49
5.2.5	Quantitative Real-Time-Polymerase Chain Reaction	50

(qRT-PCR)

6 Summary and Conclusion

56

References

Annexure

LIST OF ABBREVIATIONS

TNBC	Triple Negative Breast Cancer
EGFR	Epidermal Growth Factor
NF- κ B	Nuclear Factor κ B
IL 1 β	Interleukin 1 β
IL 6	Interleukin 6
TNF α	Tumour necrosis Factor α
HER 2	Human Epidermal growth factor Receptor 2
BRCA 1	BReast CAncer gene 1
BRCA 2	BReast CAncer gene 2
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MDA MB 231	MD Anderson - Michigan Cancer Center Breast Cancer 231
FDA	Food and Drug Administration
DPPH	2,2-diphenyl-1-picrylhydrazyl

LIST OF FIGURES

FIGURE NO	FIGURE NAME	PAGE NO
1.1	Tumour Development	1
2.1	Different pathways that involves EGFR activation	11
2.2	EGFR in Lipid raft-caveolae Endocytosis pathway	16
4.1	Structure of EGFR	21
4.2	3D and 2D structures of Nystatin	22
4.3	MDA MB 231 Cell Line (after treatment)	24
4.4	Dissolved Drug - Nystatin	25
4.5	MTT Assay for Control	27
4.6	MTT Assay for Cancer Cells	27
4.7	Forward and Reverse Primers	31
4.8	Thermocycler	32
5.1	Survival Analysis of EGFR using UALCAN	34
5.2	Survival Analysis of EGFR using KM plotter	36
5.3	Survival Analysis of EGFR using GEPIA	37
5.4	Various properties of Nystatin	42
5.5	Toxicity properties of Nystatin	43
5.6	3D structure of the Docked molecules	44
5.7	2D structure of the Docked molecules	44
5.8	Inhibition plot for Normal Cells	45
5.9	Inhibition plot for Cancer cells	46

5.10	Inhibition Comparison between Normal and Cancer cells	46
5.11	Control (Acridine Orange); Control (Ethidium Bromide); Control (Acridine Orange + Ethidium Bromide)	47
5.12	Treated (Acridine Orange); Treated (Ethidium Bromide); Treated (Acridine Orange + Ethidium Bromide)	48
5.13	Graphical Representation of AO/EB Staining results	48
5.14	DPPH Assay	49
5.15	Phase Separation during RNA Isolation	50
5.16	cDNA conversion	50
5.17	Expression of TNF α	52
5.18	Expression of IL 6	52
5.19	Expression of IL 1 β	52
5.20	Expression of NF κ B	52
5.21	Expression of BCL 2	53
5.22	Expression of BAX	53

LIST OF TABLES

TABLE NO	TABLE NAME	PAGE NO
4.1	List of Cell lines used	24
4.2	List of Primers used for qRT-PCR analysis	30
4.3	List of tests used for Statistical Analysis	33
5.1	Lipophilicity	38
5.2	Water Solubility	39
5.3	Pharmacokinetics Parameters	40
5.4	Drug-likeness	41
5.5	Medicinal Chemistry Properties	42
5.6	Toxicity	43
5.7	Binding scores for the docked molecule	44
5.8	Fold Change for various genes (TNF α , IL 6, IL 1 β , NF κ B, BCL 2, BAX)	51

CHAPTER 1

INTRODUCTION

Breast cancer is a major global health concern, characterised by the uncontrolled proliferation of abnormal cells in breast tissue. Its prevalence continues to rise, posing a substantial burden on healthcare systems worldwide. Understanding the intricacies of breast cancer, including its prevalence, risk factors, symptoms, treatment modalities, and challenges, is crucial for developing effective therapeutic strategies.

1.1 BREAST CANCER

Breast cancer is among the most commonly diagnosed cancers globally, with varying incidence rates among different populations. It is currently the second leading cause of mortality in women aged from 20 to 50 years worldwide. The majority of instances are sporadic and influenced by many risk factors, with genetic predisposition such as mutations in the BRCA1 or BRCA2 genes accounting for only a fraction of cases. These include age, gender, family history, hormonal factors, lifestyle choices (e.g., diet, physical activity), reproductive history, and environmental exposures. Recognizing symptoms such as palpable breast lumps, changes in breast size or shape, nipple abnormalities, or skin changes is crucial for early detection and prompt intervention. Despite advances in early detection, approximately 30% of patients with early-stage breast cancer still relapse with comprehensive breast cancer treatment

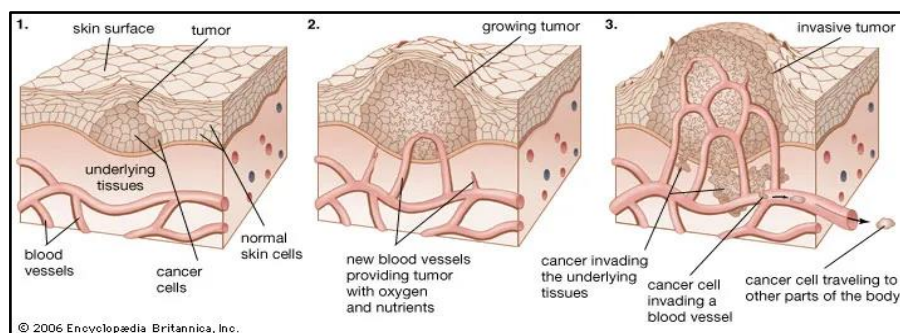


Figure 1.1 Tumour Development

1.2 TREATMENTS FOR BREAST CANCER

Breast cancer treatment involves a variety of approaches, including hormone therapy, targeted therapy, immunotherapy, chemotherapy, radiation therapy, and surgery. The cancer subtype, stage, molecular features, and patient preferences are some of the variables that influence the choice of treatment plan. The first line of treatment for a localized tumor is surgery, which can involve a mastectomy or a lumpectomy. Adjuvant treatments include hormone therapy, radiation therapy, and chemotherapy to either eradicate cancer cells that are left over after the initial treatment, reduce the likelihood of a recurrence, or alleviate symptoms. Examples of targeted medications that precisely target molecular anomalies like hormone receptor positive or HER2 overexpression are monoclonal antibodies and small molecule inhibitors. Immunotherapy is a potentially effective therapeutic option because it uses the immune system to identify and destroy cancer cells. Despite advances in treatment means, challenges such as treatment resistance, disease relapse, and adverse effects persist, underscoring the need for innovative therapeutic approaches.

1.3 TRIPLE NEGATIVE BREAST CANCER (TNBC)

Triple-negative breast cancer (TNBC) represents a distinct subtype characterised by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression and their absence makes the tumour grow quickly and spreads very faster. TNBC accounts for approximately 10-15% of all breast cancer cases and is associated with a more aggressive clinical course and poorer prognosis compared to other subtypes. Limited targeted treatment options for TNBC pose significant challenges in disease management as they are considered the most aggressive form of Breast cancer and chemotherapy remains the mainstay of treatment for TNBC. But resistance and treatment-related toxicities are common.

1.4 EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

Epidermal Growth Factor Receptor (EGFR) is a key player in cancer progression, promoting cell proliferation, survival, invasion, and metastasis. It is also an important component of the caveolae dependent Endocytosis pathway. The overexpression of EGFR in TNBC has emerged as a potential therapeutic target, yet efforts to exploit this target have faced challenges, including the development of resistance mechanisms. In TNBC, EGFR overexpression is associated with aggressive tumour behaviour and resistance to conventional therapies. EGFR signalling intersects with various inflammatory pathways, including nuclear factor-kappa B (NF- κ B), tumour necrosis factor alpha (TNF- α), interleukin 6 (IL-6), and interleukin 1 beta (IL-1 β), creating a pro-inflammatory microenvironment conducive to tumour growth and immune evasion. Dysregulation of these pathways contributes to disease progression, angiogenesis, and resistance to treatment, highlighting their significance as potential therapeutic targets.

1.5 REPURPOSING OF DRUGS

Drug repurposing, also known as drug repositioning or drug reprofiling, involves identifying new therapeutic indications for existing drugs beyond their original intended use. This approach offers several advantages, including reduced development timelines, lower costs, established safety profiles, and known pharmacokinetics. Nystatin, a polyene antifungal drug, has garnered attention for its potential anticancer properties beyond its antifungal activity. Nystatin exerts its antifungal effects by binding to ergosterol in fungal cell membranes, disrupting membrane integrity and inducing cell death. This study suggest that Nystatin, an inhibitor of lipid raft dependent caveolae endocytosis pathway, may also modulate EGFR signalling and downstream inflammatory pathways and thus is tamed as an attractive candidate for drug repurposing.

Here two major classes of approach were used: Bioinformatics and *In Vitro* approaches. Bioinformatics tools play a crucial role in drug discovery and development by facilitating the analysis of complex biological data and identifying

potential drug targets and mechanisms of action. Gene expression studies provide insights into the effects of drugs on gene expression profiles, signalling pathways, and cellular processes. Leveraging bioinformatics approaches, this thesis aims to elucidate the impact of Nystatin on EGFR expression and its downstream effects on NF- κ B, TNF- α , IL-6, and IL-1 β in TNBC models. By integrating computational analyses with experimental data, this study seeks to uncover the molecular mechanisms underlying Nystatin's anticancer effects and its potential as a therapeutic agent in TNBC may have the potential to modulate EGFR signalling and inflammatory pathways in TNBC, holds promise as a repurposed drug for cancer therapy.

Through interdisciplinary research integrating bioinformatics, molecular biology, and clinical studies, this thesis aims to contribute to the growing body of knowledge on breast cancer progression, treatment challenges, and the potential of drug repurposing to address unmet clinical needs. This thesis provides a comprehensive examination of breast cancer, with a particular emphasis on treatment challenges and the potential repurposing of Nystatin, an FDA-approved drug with emerging anticancer properties.

CHAPTER 2

LITERATURE SURVEY

2.1 BREAST CANCER

Breast cancer represents a complex disease attributed by the dysregulated proliferation of cells within the breast tissue, often forming a tumour or lump, with the potential to metastasize to other parts of the body, particularly the lymph nodes. It remains the most common cancer among women in India, posing a huge health burden. [World Health Organization: WHO 2024]. Globally, it ranks high among malignant tumours and remains a leading cause of mortality in women aged 20 to 50 years, with its incidence steadily increasing in recent years. Breast cancer has a complex aetiology that includes genetic, hormonal, psychological, and environment-related factors. Studies have identified various risk factors associated with its development, including socioeconomic status, lifestyle habits such as smoking and physical inactivity, stress, and dietary patterns [Lacoviella, L. *et al.*, 2020]. Notably, women are significantly more susceptible to breast cancer compared to men, with hormonal changes playing a crucial role in its genesis. Genetic predisposition also contributes to the risk of breast cancer, with approximately 5-6% of cases attributed to hereditary factors. Mutations in specific genes, such as BRCA-1 and BRCA-2, account for a significant portion of hereditary breast cancer cases, highlighting the importance of genetic testing and counselling for high-risk individuals [Richje, R.C. and Swanson, J.O. 2003].

In India, breast cancer incidence continues to rise, comprising a substantial proportion of all female cancers. From 1990 to 2016, the age-standardised incidence rate of female breast cancer grew by 39.1%, which is a troubling trend. [Sathishkumar, K. *et al.*, 2024]. By 2022, it is estimated that there will be approximately 216,108 new cases of breast cancer in India, further emphasising the urgent need for comprehensive prevention, early detection, and treatment strategies [Malvia, S. *et al.*, 2017].

Breast cancer is not an unitary disease, but rather an array of subtypes with diverse genomic characteristics and clinical behaviours. Breast cancer can be divided into four subtypes:

1. Luminal A.
2. Luminal B.
3. HER2-positive.
4. Triple-negative.

Luminal A tumours usually express the oestrogen receptor (ER) and/or the progesterone receptor (PR), have modest proliferation rates, and have the best prognosis among breast cancer subtypes. Luminal B tumours, on the other hand, are more advanced, have a greater proliferation rate, and have a worse prognosis than Luminal A tumours. HER2-positive breast cancer is distinguished by overexpression of the HER2/neu protein, which is linked with aggressive behaviour and rapid development. TNBC is characterised by its aggressive behaviour, early relapse, and absence of expression of ER, PR, and HER2 [Orrantia-Borunda, E. *et al.*, 2022]. The survival rates for individuals with various breast cancer subtypes range dramatically. Patients with the Luminal A subtype had the greatest survival rates, followed by the Luminal B, HER2-positive, and TNBC subtypes, which have the lowest survival results. The differences in survival rates are mostly due to each subtype's unique biological traits and clinical behaviours. Metastasis, or the spread of cancer cells to distant organs, is an ongoing concern for breast cancer patients and has a substantial influence on their prognosis [Female Breast Cancer Subtypes - Cancer Stat Facts]. According to study findings, certain subtypes are more likely to spread to certain metastatic areas. For example, Luminal A and Luminal B subtypes are more likely to metastasize to bone, whereas HER2-positive and TNBC subtypes are more likely to metastasize to visceral organs such as the liver, lung, and brain [Pulido, C. *et al.*, 2017].

Furthermore, the chance of acquiring TNBC is especially high in breast cancer patients due to its aggressive nature and restricted treatment options. TNBC accounts for around 20% of all breast cancers and is linked to younger age, African-American ancestry, and genetic risk, including mutations in the BRCA1 and BRCA2 genes. Patients with TNBC are more likely to experience early

recurrence and metastasis, emphasising the importance of specific therapy methods and constant monitoring in this population [Obidiro, O. *et al.*, 2023].

There are various personalised treatment options available and they are opted according to the detected stage and type of Breast Cancer (BC). The commonly used treatments for all BC types includes surgery, radiotherapy, and chemotherapy as they form the cornerstone of BC treatment, addressing both localised and systemic aspects of the disease. Surgical interventions encompass mastectomy or lumpectomy, with axillary lymph node dissection or sentinel lymph node biopsy aiding in staging and therapeutic decision-making. Lumpectomy, coupled with adjuvant radiotherapy, offers comparable outcomes to mastectomy, highlighting the importance of personalised treatment approaches tailored to patient and tumour characteristics. Radiotherapy, administered post-surgery, plays a pivotal role in reducing recurrence risk and BC mortality, particularly in high-risk patients. Techniques to minimise cardiopulmonary toxicity, such as prone positioning and intensity-modulated radiotherapy, are employed to optimise treatment outcomes. However, challenges like radiation resistance, attributed to hypoxic tumour microenvironments and cancer stem cells, underscore the need for further research into novel therapeutic strategies [Burguin, A. *et al.*, 2021].

The integration of surgery, radiotherapy, chemotherapy, and targeted therapies underscores the importance of a multidisciplinary approach in managing BC, emphasising the need for personalised treatment strategies tailored to individual patient characteristics and tumour biology [Almeida-Ferreira, C. *et al.*, 2024].

2.2 RESISTANCE TO CHEMOTHERAPY IN BREAST CANCER

Chemotherapy remains a mainstay in BC treatment, comprising cytotoxic agents like anthracyclines, taxanes, and alkylating agents established as the sole systemic treatment option for Triple Negative Breast Cancer as it is the most lethal subtype of BC due to its convoluted heterogeneity, aggressive nature, rapid growth rate, increased risk of metastasis, high recurrence risk, and scarcity of therapeutic choices. When compared to the luminal A and luminal B subtypes,

TNBC has the lowest survival rate. Chemotherapy has the ability to downstage tumours and can be administered based on the patient's reaction and individual needs [Nedeljković, M. and Damjanović, A., 2019]. Even Though chemotherapy is solely used, it does pose some resistance threat during treatment of BC as these tumours may become resistant to cytotoxic drugs. Numerous mechanisms can lead to the development of chemoresistance amongst which the significant ones are:

- ABC transporters.
- Cancer stem cells.
- Hypoxia.
- Avoidance of apoptosis.
- Signalling pathways.

These are implicated in TNBC chemoresistance and progression [Britton, K.M. *et al.*, 2012].

Chemotherapy resistance in TNBC is significantly influenced by ABC transporters, specifically ABCC1, ABCG2, and ABCC11. They impede the efficacy of treatment by effluxing different medicines. Although it can be difficult to suppress their expression or activity, new approaches have promise. For example, NSAIDs sensitise resistant cells, and PZ-39 quickens the breakdown of transporters. Furthermore, TKIs can inhibit them at high concentrations through their role as transporter substrates, which may improve chemotherapy. Preclinical evidence suggests ABC transporter inhibition in TNBC, despite the paucity of clinical data. This opens up a viable route for improved treatment approaches [Britton, K.M. *et al.*, 2012]. In solid tumours, including TNBC, cancer stem cells (CSCs) are essential for tumour renewal and chemoresistance. Increased CSC presence in residual tumours after chemotherapy suggests that these cells survive on a selective basis and may play a role in tumour recurrence. Due to its limited therapeutic choices, TNBC poses a substantial problem given its intrinsic enrichment in CSCs. CSCs are resistant to cytotoxic treatments because they express large quantities of ABC transporters, particularly ABCG2. They also exhibit dormancy. Targeting CSCs has attracted a lot of attention. Furthermore, blocking signalling pathways like TGF- β , Notch, Wnt/ β -catenin, and Hedgehog that

are essential for CSC self-renewal appear promising. These pathways are linked to metastasis, chemoresistance, and tumorigenesis in TNBC. Nevertheless the preclinical achievements, obstacles persist in converting these discoveries into therapeutic interventions, such as possible toxicity and intricate interactions among signalling pathways. However, focusing on CSCs and the pathways that are linked to them offers a promising strategy to enhance the results of TNBC treatment [Britton, K.M. *et al.*, 2012].

Triple-negative breast cancer (TNBC) chemoresistance is mostly dependent on signalling pathways, which include those involving NF- κ B, PTEN/PI3K/AKT/mTOR, JAK/STAT, and receptor tyrosine kinases. NF- κ B is a prime target for therapy because it inhibits apoptosis and promotes the progression of TNBC. Despite the fact that several inhibitors have been created, toxicity and pleiotropic effects make clinical translation difficult. Aggressive behaviour and chemoresistance in TNBC are partly caused by hyperactivation of the PI3K/AKT/mTOR pathway resulting from PTEN loss. In TNBC, pro-inflammatory cytokines such as IL6 and IL8 trigger the JAK/STAT pathway, which in turn promotes tumorigenesis and chemoresistance. TNBC chemoresistance is also associated with receptor tyrosine kinases, including EGFR and IGF-1R. Targeted medicines against EGFR and IGF-1R are being studied despite obstacles, with a move toward combination approaches to increase efficacy [Changavi, A.A. *et al.*, 2015]. Therefore, a better treatment strategy can be brought out by targeting these signalling pathways that hold great promise to overcome chemoresistance.

2.3 ROLE OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) IN BREAST CANCER

Epidermal Growth Factor Receptor (EGFR) also known as ErbB1 and HER1, is a transmembrane protein belonging to the receptor tyrosine kinase (RTK) family as well as the EGFR family. The human EGFR family comprises of four transmembrane glycoproteins that share extracellular ligand-binding and intracellular receptor tyrosine kinase domains. Many biological processes are impacted by the activation of pathways such as PI3 kinase, Ras-Raf-MAPK, JNK,

and PLC γ by EGFR signalling. Ligands change adhesion and motility, stimulate proliferation, and stop apoptosis. Breast epithelial cells scatter and invade when EGFR is activated, which also stimulates angiogenesis. Dysregulation of EGFR pathways is associated with a poor prognosis and resistance to targeted therapy because of cross-talk with other receptor tyrosine kinases. It plays a pivotal role in regulating cellular processes such as cell proliferation, survival, and differentiation [Masuda, H. *et al.*, 2012].

They are one of the most notable molecular targets identified for targeted therapy. Their overexpression is associated with EGFR expression, which occurs in 15-45% of breast cancers, is most common in basal-like carcinomas but can also be found in HER-2/neu positive or luminal B types. EGFR positive is frequently seen in younger patients and is associated with overexpression of HER-2/neu, greater proliferation, decreased hormone receptor levels, and genomic instability. Its existence implies a worse prognosis and increased relapse risk even with adjuvant therapy. EGFR expression is common in triple-negative breast cancer (TNBC) and has a bad prognosis, displaying basal markers and medullary carcinomas. It has an inverse relationship with the expression of hormone receptors and is also linked to youth. Aggressive tumour features include a higher grade, a medullary carcinoma subtype, and a negative ER/PR status are all correlated with EGFR positive. Studies differ, nonetheless, in their correlation with lymph node involvement. Furthermore, there are connections between EGFR expression and lymphocytic infiltration and tumour necrosis, but not with characteristics like pushing boundaries or periductal elastosis. These results demonstrate the importance of EGFR in characterising aggressive subtypes of breast cancer and its potential as a target for therapy [Changavi, A.A. *et al.*, 2015].

2.4 ROLE OF VARIOUS SIGNALLING PATHWAYS IN BREAST CANCER PROGRESSION

Cell signalling pathways serve an important role in breast cancer genesis and progression. Mutations in oncogenes, hyperactive proteins, or inactivation of tumour suppressor genes are frequently the cause of dysregulated pathways.

Many signalling pathways, such as those mediated by apoptotic processes, hypoxia-inducible factors, and calcium-sensitive receptors, have been linked to breast cancer. However, the human epidermal growth factor type-2 (HER2/Neu or c-ErbB2) and estrogen receptor (ER) are the most researched pathways in breast cancer. The significance of signal integration and transduction in the pathophysiology of breast cancer is highlighted by the activation of downstream pathways by these receptors, including the PI3K/Akt/mTOR pathway, glycogen synthase kinase-3 (GSK-3), and mitogen-activated protein kinases (MAPKs) [Ortega, M.A. *et al.*, 2020]. A few important pathways are enlisted below.

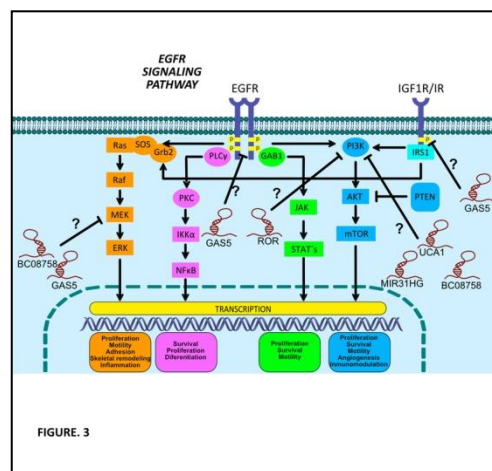


Figure 2.1 Different pathways that involves EGFR activation

2.4.1 Nuclear Factor- κ B (NF- κ B)

Nuclear Factor kappa B (NF- κ B), a key factor in inflammatory signalling, is linked to cancer formation. Both canonical (NEMO-dependent) and non-canonical (NEMO-independent) pathways are used in its operation. RelA and RelB transcription factor activation results from NEMO activation by cytokines such as TNF and IL-1, which is a step in the canonical pathway activation process. NIK-mediated TNF receptor activation is a necessary step in NEMO-independent non-canonical pathway activation. A number of carcinomas, including bladder, lung, and ovarian cancer, have different tumour microenvironments and prognoses due to the role of NF- κ B on cell survival and proliferation [Devanaboyina, M. *et al.*, 2022]. The multifaceted actions of NF- κ B in tumour progression include inducing

an inflammatory tumour microenvironment (TME) and modulating the production of cytokines. Its activation plays a role in leukocyte infiltration and altered cell death in breast cancer. In particular, NF- κ B may be a useful diagnostic and therapeutic target for overcoming chemotherapy resistance in BC that has spread to other locations [Garlanda, C. *et al.*, 2013].

2.4.2 Interleukin 1B (IL 1B)

Interleukin 1 B is a cytokine that belongs to the IL-1 family. IL-1 β , produced by hematopoietic cells such as monocytes and macrophages, is crucial for inflammation. Unlike IL-1 α , IL-1 β cannot function until caspase-1 cleaves it, a process that is frequently aided by the NLRP3 inflammasome. Autoinflammatory disorders can arise from mutations in NLRP3, as they cause an overabundance of IL-1 β production. Beyond immune control, IL-1 β causes tumour angiogenesis and metastasis, greatly enhancing the inflammatory milieu in tumours and initiating primary carcinogenesis [Garlanda, C. *et al.*, 2013]. Human breast cancers have IL-1 β expression, which correlates with neoplasm initiation and development. IL-1 β overexpression affects breast cancer development in a number of ways. Via the transglutaminase 2/NF- κ B pathway, it increases the production of IL-6 and increases the aggressiveness of luminal-type breast cancer cells. Furthermore, through FGFR1 signalling, IL-1 β promotes COX-2 production, which aids in the development of early-stage mammary lesions. Studies using defective mice or anti-IL-1 antibodies have shown promise in reducing the evolution of breast tumours by the inhibition of IL-1 β , either directly or through downstream effectors like IL-6 [R    , C. and Ghiringhelli, F., 2020].

2.4.3 Interleukin 6 (IL 6)

The glycoprotein IL-6, weighing 26 kDa, has been shown to have both pro- and anti-inflammatory properties. It communicates via a cell-surface type-I receptor complex made up of gp130 and the membrane-bound IL-6 receptor (IL-6R). There are two types of IL-6R: soluble (sIL-6R) and membrane-bound (mIL-6R). IL-6 selectively activates pro- and anti-inflammatory responses by activating trans-signalling via sIL-6R and classical signalling via mIL-6R. The many biological

effects of IL-6 are mediated by three signalling pathways: the JAK/STAT3, Ras/Raf/MAPK, and PI3K/Akt pathways [Chen, J. *et al.*, 2022].

They regulate the inflammation and enhance monocyte infiltration at the inflammatory site. IL-6 responsive tissues would become resistant gradually during chronic inflammation, which correlated with high basal level of IL-6. IL-6 was also elevated in many solid tumours including breast cancer, which correlated with poor prognosis and metastasis. At the site of inflammation, they simultaneously regulate inflammation and promote monocyte infiltration. Chronic inflammation led to the progressive resistance of IL-6-responsive tissues, which was associated with a high basal level of IL-6. Additionally, IL-6 was found to be high in a number of solid tumours, such as breast cancer, and this was linked to a poor prognosis and metastasis [Scheller, J. and Rose-John, S., 2006].

2.4.4 Tumour Necrosis Factor α (TNF α)

TNF- α is a key cytokine involved in immunological and inflammatory responses. It is rapidly transcribed in several cell types when exposed to infections, inflammation, and stress. Numerous stimuli, including calcium signalling, pathogens, mitogens, chemical stress, radiation, and receptor ligands, can activate its transcription. TNF- α plays a vital function in both innate and adaptive immunity due to its wide participation in immune regulation, which spans multiple cell types such as neutrophils, dendritic cells, lymphocytes, monocytes, macrophages, and fibroblasts [Falvo, J.V. *et al.*, 2010]. TNF α plays a complex role in TNBC, exhibiting pro-apoptotic and pro-malignant actions based on the specific cellular environment. It can cause apoptosis in some TNBC cell lines by downregulating anti-apoptotic genes and activating c-Jun, but it also encourages tumour growth by triggering the p38/MAPK and NF- κ B pathways, which activate STAT3 and cause the production of HBXIP. Furthermore, TNF α promotes cancer cell motility, invasion, and epithelial-mesenchymal transition (EMT) via a number of signalling pathways, including PI3K/Akt and p42/p44 MAPK [Mercogliano, M.F. *et al.*, 2020].

2.4.5 BCL 2 Family

The Bcl-2 family of proteins controls both apoptosis and mitochondrial outer membrane permeabilization (MOMP). It includes pro-apoptotic (Bak, Bax), anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1), and sensitising (Bad, Noxa) proteins. The pathophysiology of breast cancer (BC) and treatment resistance are influenced by the dysregulation of these proteins. Anti-apoptotic Bcl-2 protein overexpression in BC improves cell survival and resistance to endocrine, targeted, and chemotherapeutic treatments (trastuzumab, for HER2+ BC, and tamoxifen, for ER+ BC). Pro-apoptotic proteins also play a role in the development and progression of BC. Bcl-2 family protein-targeting treatment approaches seek to overcome resistance mechanisms and reestablish apoptosis, underscoring their significance as possible targets for therapeutic intervention in the management of bladder cancer. Treatment approaches and the biology of breast cancer can be better understood by taking into account the complex balance of Bcl-2 family proteins [Rai, A. *et al.*, 2022].

2.5 NYSTATIN AS A DRUG

Nystatin is a polyene antifungal drug generated from the bacteria *Streptomyces noursei*, which is found in soil. It is an antibiotic that belongs to the polyene class, which is distinguished by a macrocyclic lactone ring that has several conjugated double bonds. Since its initial isolation by Rachel Fuller Brown and Elizabeth Lee Hazen in 1950, nystatin has shown to be a vital weapon in the fight against fungal infections, especially those brought on by *Candida* species. Nystatin is mostly used as a fungicidal drug to treat infections brought on by *Candida albicans*, an opportunistic fungus, including vaginal yeast infections and oral thrush (oral candidiasis) [Hamilton-Miller, J.M., 1973].

It works well against some fungal strains that cause cutaneous infections as well as other species of *Candida*. By attaching to ergosterol, a component of fungal cell membranes, nystatin creates pores and compromises the integrity of the membrane, hence exhibiting its antifungal properties. Fungal cell death results from this disturbance, which also causes internal components like potassium and

other ions to flow out. Notably, nystatin has little systemic toxicity and is therefore appropriate for treating localised fungal infections due to its poor absorption when applied locally or orally. Because of its low toxicity profile and broad-spectrum antifungal activity, nystatin is still a mainstay in the treatment of a variety of fungal infections, especially those that impact the skin and mucous membranes [Rai, A. *et al.*, 2022]. Nystatin is available in various forms and concentrations, including tablets, troches, suspensions, creams, and ointments, under brand names like Mycostatin and Nystop. The typical dose for oropharyngeal candidiasis ranges from 500,000 to 1,000,000 units, taken orally 3 to 5 times daily for 1 to 2 weeks. Common side effects include metallic taste, dry mouth, and nausea. While rare, nystatin therapy has been minimally associated with serum enzyme abnormalities, but acute hepatic injury cases remain unsubstantiated, affirming its overall safety profile [(U.S.), N.I. of D. and D. and K.D., 2012].

2.6 NYSTATIN AS AN INHIBITOR OF LIPID RAFT-CAVEOLAE ENDOCYTOSIS PATHWAY

The lipid raft-caveolae endocytosis pathway is crucial for cellular uptake of extracellular and signalling molecules, including epidermal growth factor receptor (EGFR). Lipid rafts, enriched in cholesterol and sphingolipids, and caveolae, invaginated lipid rafts with caveolin proteins, facilitate this process. EGFR, a receptor tyrosine kinase, is often localised within these specialised membrane microdomains, where it participates in signalling cascades regulating cell proliferation, survival, and migration. Dysregulation of lipid raft-caveolae endocytosis, including EGFR internalisation, has implications in cancer progression and metastasis [Quest, A.F.G. *et al.*, 2004]. Overexpression of caveolin-1, a key component of caveolae, is frequently observed in various cancers and is associated with aggressive tumour behaviour, often coinciding with EGFR activation. The interaction between EGFR and caveolae can modulate downstream signalling pathways, contributing to tumour growth and metastasis. Understanding the intricate relationship between EGFR localization and lipid raft-caveolae endocytosis provides valuable insights into cancer biology and offers potential avenues for targeted therapeutic interventions aimed at disrupting

aberrant signalling pathways and impeding tumour progression [Williams, T.M. and Lisanti, M.P., 2004].

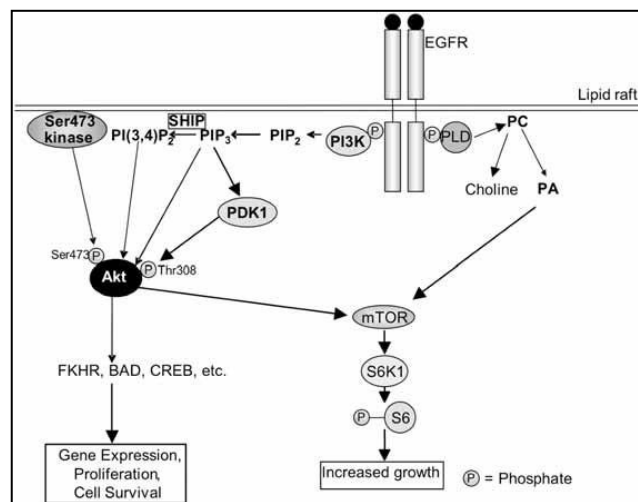


Figure 2.2 EGFR in Lipid raft-caveolae Endocytosis pathway

Nystatin, a cholesterol-sequestering agent, has been shown to selectively disrupt caveolae/lipid raft-dependent endocytosis without affecting clathrin-mediated internalisation. It increases the internalisation of molecules in endothelial cells. Despite its cholesterol-sequestering action, nystatin treatment does not compromise cell viability substantially. Interestingly, nystatin switches the internalisation pathway of certain molecules from caveolae/lipid rafts to clathrin-coated pits, enhancing their uptake efficiency. This phenomenon suggests a potential role for nystatin in modulating lipid raft caveolae endocytosis and may have implications for various cellular processes and therapeutic strategies [Chen, Y. *et al.*, 2020].

2.7 REPURPOSING OF DRUGS IN CANCER

Repurposing, or repositioning, pharmaceuticals refers to the process of finding new therapeutic applications for already-approved medications for different ailments. It has benefits include lowering development delays, utilising drugs with proven safety profiles, and offering cost and time efficiency. Repurposed medications frequently have a variety of modes of action, making it possible to

target several pathways implicated in the development of disease. This tactic increases the range of available treatments, especially for illnesses for whom there are no viable treatments or where drug resistance is an issue. Combining used drugs with existing treatments can improve efficacy and overcome resistance, resulting in synergistic benefits. Additionally, drug repurposing reveals new therapeutic targets and pathways while offering insights into disease mechanisms [Attard, G. *et al.*, 2012].

Evaluating the potential of antifungal drugs is one exciting direction in the field of drug repurposing for cancer treatment. Although the antifungal drugs are mostly intended to treat fungal infections, some of them have also shown signs of anticancer activity, which makes them appealing candidates for other uses. Ketoconazole is a famous example. Through a number of routes, the commonly used antifungal drug ketoconazole has demonstrated encouraging anticancer effects. Its capacity to suppress steroid biosynthesis-related enzymes has been demonstrated in studies [Attard, G. *et al.*, 2012]. This results in lower levels of hormones like cortisol and testosterone, which can promote the formation of several malignancies, including prostate cancer. Furthermore, ketoconazole has shown antiangiogenic qualities, which inhibit the development of new blood vessels, which are essential for tumour growth and metastasis [Goldstein, D.A. *et al.*, 2015]. Additionally, ketoconazole has anti-proliferative properties by causing cancer cells to undergo apoptosis and interfering with the advancement of the cell cycle. The effectiveness of ketoconazole as a stand-alone treatment or in conjunction with other anticancer drugs has been investigated in a number of clinical trials and preclinical investigations. Although further investigation is required to completely understand its modes of action and determine the best dosage schedules, the use of ketoconazole and other antifungal medications in the treatment of cancer shows great potential [A Chen, S.C. *et al.*, 2005].

CHAPTER 3

AIM AND SCOPE

3.1 AIM

To repurpose the FDA-approved antifungal Nystatin as a breast cancer treatment drug by studying its inhibitory effects on NFkB protein and various other cytokines production via interactions with the cell surface receptor Epidermal Growth Factor Receptor (EGFR) utilising both *In Silico* and *In Vitro* techniques.

3.2 SCOPE

- To use molecular docking methods, study the possible binding interactions between Nystatin and EGFR to predict the affinity and manner of inhibition on NFkB protein production in breast cancer cells.
- To conduct MTT tests and staining assays to evaluate the influence of Nystatin on breast cancer cell viability.
- To analyse the gene expression levels related with NFkB signalling and other cytokines using quantitative polymerase chain reaction (qPCR).

This project aims to use the FDA-approved antifungal Nystatin to treat breast cancer by inhibiting NFkB protein synthesis and cytokines through interactions with HER2, ER, and TNF- α receptors. The initiative uses molecular docking to predict Nystatin's binding to TNF Receptor and evaluates its affinity and method of NFkB inhibition. Nystatin's effect on breast cancer cell viability was evaluated using MTT tests and staining assays, as well as qPCR analysis of NFkB signalling and cytokine gene expression. The study, which combines computational modelling and experimental validation, seeks to understand Nystatin's therapeutic potential in breast cancer treatment.

CHAPTER 4

MATERIALS AND METHODS

4.1 IN SILICO STUDIES

4.1.1 Pan-cancer survival analysis of EGFR using TCGA

Samples from UALCAN's The Cancer Genome Atlas (TCGA) were used to analyze the survival of patients expressing EGFR (RRID SCR 015827). An interactive web tool called UALCAN (helps with the detailed examination of TCGA survival analysis data. Through the gateway, researchers can examine the relative expression of one or more genes in tumors (including those with varying stages, grades, and subtypes) and normal samples. They can also investigate how the expression levels affect overall survival (OS) and pinpoint the up- and down-regulated genes in certain cancers. With the time in days represented on the x-axis, the survival probability was plotted on the y-axis and compared for normal versus tumor samples. P-values below 0.05 were regarded as significant.

4.1.2 Pan-cancer survival analysis of EGFR using KM

The Kaplan–Meier Plotter database (<https://kmplot.com/analysis/>), an online resource with clinical and gene expression data, was utilized to evaluate the effects of several genes on the survival of 15 distinct cancer types. There are now 3452 lung, 1440 stomach, 2190 ovarian, and 6234 breast cancer datasets in this database. Significantly, this database draws its data from TCGA (The Cancer Genome Atlas) and the European Genome-phenome Archive (EGA), in addition to GEO. Using the Kaplan–Meier plotter database, the predictive relevance of EGFR mRNA expression in triple negative breast cancer was assessed; the log rank P-value and hazard ratio with 95% confidence intervals were also produced.

4.1.3 Validation of EGFR survival analysis study in pan-cancer

The results from above studies were validated using Gene Expression Profiling Interactive Analysis (GEPIA, 2017). Key interactive and programmable features offered by GEPIA include differential expression analysis, profiling charting, correlation analysis, patient survival analysis, related gene detection, and dimension reduction analysis. GEPIA bridges the gap between large data generated by cancer genomics and the transmission of integrated information to end users. All data was set to a default p-value of 0.05. The y-axis was set to percentage of survival, which was plotted for normal and tumour samples, with the x-axis set to time in months.

4.1.4 In Silico Characterization of Nystatin

a) Pharmacokinetics and Drug-likeness Predictions

Tools like SwissADME (<http://www.swissadme.ch/>) and Molsoft L.L.C (<https://molsoft.com/mprop/>) were used to predict pharmacokinetic characteristics and drug-likeness in small compounds. SwissADME, a web-based tool, offers insights into essential properties such as lipophilicity, water solubility, medicinal chemistry attributes, and the potential for P-glycoprotein substrate activity, aiding in effective drug development. On the other hand, Molsoft is used to forecast a drug's molecular characteristics and assess its drug-likeness. These tools play a crucial role in optimising drug candidates by providing valuable information on their pharmacokinetic profiles and molecular features, contributing to the rational design and development of pharmaceutical agents with improved chances of success in clinical settings.

b) Toxicological Predictions

To assess the drug's toxicological parameters, the online ProTox-II server (https://tox-new.charite.de/protox_II/) was employed. Functioning as a virtual lab, ProTox-II predicts toxicity levels for chemical and small bioactive compounds. This tool serves as a valuable resource for researchers and drug developers, offering

insights into potential toxic effects, aiding in the evaluation and optimization of drug candidates for safety and efficacy before advancing them further in the development process.

4.1.5 Protein Preparation

The three-dimensional crystallographic structures of target receptor proteins Epidermal Growth Factor Receptor (EGFR) were obtained from the Protein Data Bank (PDB) at <https://www.rcsb.org/>. Subsequently, the collected 3D structures underwent refinement using Molegro Software, which eliminated extraneous protein chains, water molecules, heteroatoms, and ligands. The processed molecules binding sites were then analysed utilising Biovia Discovery Studio 2017. To gain insights into the spatial arrangement of amino acid residues associated with the proteins, the binding sites were visualised using a Ramachandran plot. This comprehensive approach enhances the understanding of the target receptors' structural characteristics, facilitating subsequent steps in drug design and development.



Figure 4.1 Structure of EGFR

4.1.6 Ligand Preparation

The two-dimensional (2D) and three-dimensional (3D) structures of Nystatin were sourced from the PubChem chemical database (<https://pubchem.ncbi.nlm.nih.gov/>). Additionally, the canonical SMILES

representation of the drug was obtained. The 3D structure was retrieved in SDF format and converted to PDB format using the OPENBABEL web tool (<http://www.cheminfo.org/Chemistry/Cheminformatics/FormatConverter/index.html>). To ensure accuracy, the structure underwent scrutiny in Molegro, where any extra molecules were meticulously checked and verified for a refined structure of Nystatin's molecular composition.

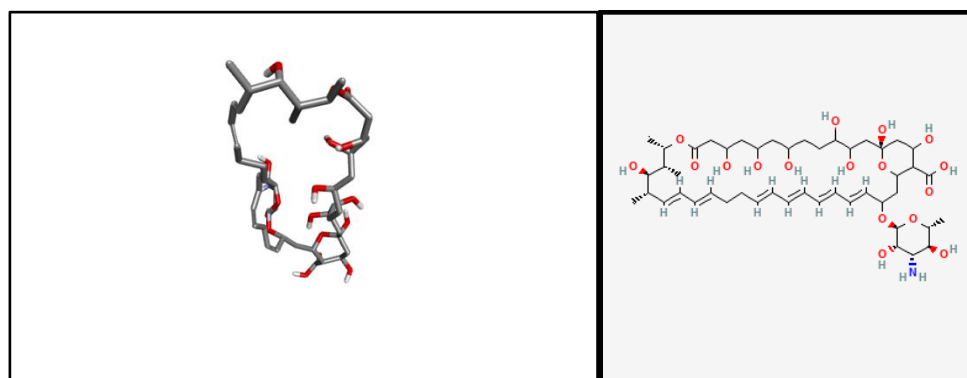


Figure 4.2 3D and 2D structures of Nystatin

4.1.7 Molecular Docking using PyRx Software

PyRx software was used for molecular docking. Pyrx is a Computational Drug Discovery virtual screening programme that can potentially be used to screen libraries of compounds against prospective therapeutic targets. They feature an easy-to-use user interface that allows users to execute virtual screening from any platform and assists them in every stage of the process, from data preparation to job submission and results analysis.

Initially, the processed protein structure and drug molecule were imported into PyRx individually. The proteins were designated as macromolecules, and the drug molecules were defined as ligands. Subsequently, energy minimization was applied specifically to the ligands. This step refines the ligands' conformation and geometry, optimising their structures for more accurate docking simulations. The refined structures of both the proteins and ligands were then converted to the PDBQT format, a specific format used by Autodock Vina, the default docking

engine in PyRx. Following this, a critical step involved placing a grid box around the predicted binding site on the protein. The grid box allows users to define the search space for docking calculations, specifying the approximate location and dimensions of the binding site. This is crucial for accurate docking predictions, ensuring that the ligand is positioned within a biologically relevant region of the protein. With the grid box in place, the docking calculations were initiated. After completion, the docked ligand structures with the best binding affinity scores (kcal/mol) were downloaded in PDB format. To gain a comprehensive understanding of the molecular interactions, the downloaded structures were then visualised in Biovia Discovery Studio 2017. This visualisation encompassed the examination of 2D and 3D structure, as well as an analysis of the bonds involved.

4.1.8 Validation Docking using iGEM Dock

To validate the findings obtained through PyRx, the iGeneric Evolutionary Method Docking (iGEM), specifically iGemdock, was utilised. iGemdock serves as a graphical environment dedicated to recognizing pharmacological interactions and facilitating virtual screening in drug discovery. This tool is instrumental in assessing the compatibility of potential drugs with proteins. It conducts a comprehensive analysis of interactions, generating profiles for electrostatic (E), hydrogen-bonding (H), and Van der Waals (V) protein-compound interactions, while also predicting their collaborative effects. As a user-friendly tool, iGemdock is freely available for non-commercial research, enhancing drug discovery efforts. The iGemdock procedure involved importing prepared protein and drug molecules in PDB file format. Docking was performed for both molecules, yielding a binding score. Subsequent to the docking process, a post-docking analysis was executed to delve deeper into the interactions and validate the results obtained through PyRx, thereby bolstering the reliability of the drug discovery findings.

4.2 IN VITRO STUDIES

4.2.1 Mammalian cell culture

The cell lines (Hela cells, MDA MB 231) were acquired from the National Centre for Cell Science (NCCS), Pune. The cells were then cultured in T-25 cm² flasks in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 100 mg/L penicillin, and 100 mg/L streptomycin at 37°C and 5% CO₂ in a humidified incubator. After reaching 80% to 90% confluence, the cells were subcultured using the trypsinization procedure. Cell monolayers were rinsed with about 2 mL of Phosphate Buffer Saline (PBS). Then, 0.5 to 2 mL was added and incubated for 1 minute in the CO₂ incubator. The solution was aspirated and centrifuged at 900 rpm for 3 minutes. The pellet was collected and reconstituted in 2 mL of DMEM media with 10% FBS. The cells were then divided into four clean sterile bottles (0.5 mL each), which were subsequently filled with DMEM medium to a volume of 2 mL.

Table 4.1 List of Cell lines used

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

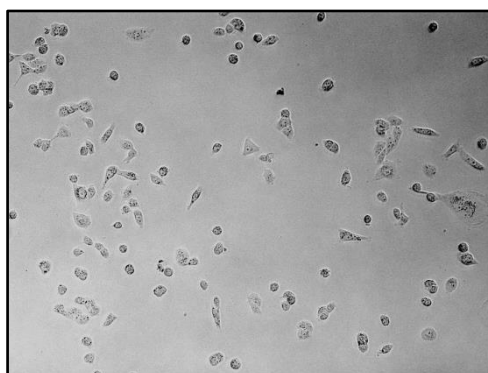


Figure 4.3 MDA MB 231 Cell Line (after treatment)

4.2.2 Preparation of Nystatin

A solution containing 1 mg of Nystatin, 1 μ L of DMSO, and 1 mL of DMEM was prepared. The suspension was then vortexed until fully dissolved. The tubes were kept at 4°C until further use.

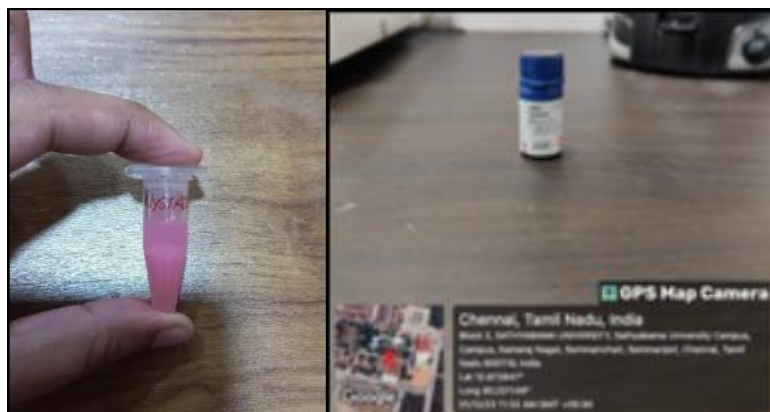


Figure 4.4 Dissolved Drug - Nystatin

4.2.3 MTT Cytotoxicity Assay

A popular method for assessing cell viability and proliferation in studies is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, or MTT assay. It is based on the fact that in metabolically active cells, mitochondrial enzymes transform MTT into formazan crystals and the absorbance of these crystals indicates the health of the cell.

a) MTT for Normal Cell Line (Control)

100 μ L containing 1×10^4 cells of the Hela cells with 90% confluency were seeded in a 96-well plate and cultured at 5% CO₂ in a humidified incubator for 24 hours. The next day, the cells were treated with dissolved drugs at the following concentrations: 2.1 μ L, 4.2 μ L, 8.4 μ L, 16.8 μ L, 21 μ L, and 33.6 μ L. The cells were cultured in a humidified incubator for 24 hours with 5% CO₂. The incubated cells were now provided to the MTT assay. To make the MTT solution, dissolve the MTT salt in PBS at a concentration of 5 mg/mL. The solution was vortexed and then filtered. It was then kept at -20°C for future use. To conduct the test, the

media from the cell cultures was discarded. Each well received 50 μL of serum-free medium and 50 μL of MTT solution. The plate was then covered in aluminium foil and incubated at 37°C for 3 hours. After incubation, remove the media and dye. Add 150 μL of DMSO (MTT solvent) to each well. Wrap the plate in foil and shake on an orbital shaker for 15 minutes. The solution was pipetted many times to thoroughly dissolve the MTT formazan. Finally, the absorbance was measured at OD = 570 nm.

b) MTT for Cancer Cell Line (Test Group)

100 μL containing 1×10^4 cells of the MDA MB 231 cells with 90% confluency were seeded in a 96-well plate and cultured at 5% CO_2 in a humidified incubator for 24 hours. The next day, the cells were treated with dissolved drugs at the following concentrations: 3 μL , 6 μL , 12 μL , 24 μL and 48 μL . The cells were cultured in a humidified incubator for 24 hours with 5% CO_2 . The incubated cells were now provided to the MTT assay. To make the MTT solution, dissolve the MTT salt in PBS at a concentration of 5 mg/mL. The solution was vortexed and then filtered. It was then kept at -20°C for future use. To conduct the test, the media from the cell cultures was discarded. Each well received 50 μL of serum-free medium and 50 μL of MTT solution. The plate was then covered in aluminium foil and incubated at 37°C for 3 hours. After incubation, remove the media and dye. Add 150 μL of DMSO (MTT solvent) to each well. Wrap the plate in foil and shake on an orbital shaker for 15 minutes. The solution was pipetted many times to thoroughly dissolve the MTT formazan. Finally, the absorbance was measured at OD = 570 nm.

$$\text{Percentage of Inhibition} = 100 - \left(\frac{\text{Control OD} - \text{Treated OD}}{\text{Control OD}} \right) \times 100$$



Figure 4.5 MTT Assay for Control

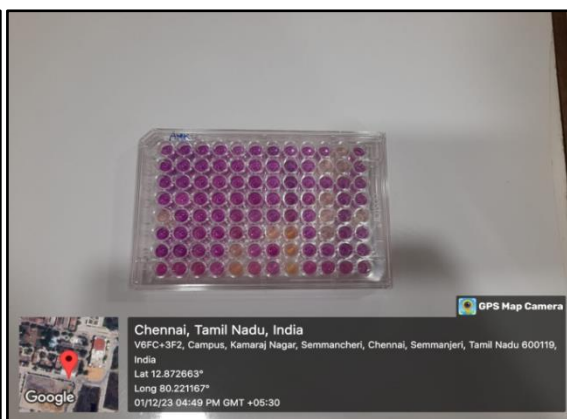


Figure 4.6 MTT Assay for Cancer Cells

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

It is used to view the apoptosis-associated changes of cell membranes during the process apoptosis. Acridine Orange (AO) is a vital dye that will stain both live and dead cells while Ethidium Bromide (EB) will only stain the cells that have lost their integrity. The live cells or cells in the stage of early apoptosis will be stained green while the cells. Late apoptotic cells will be stained with orange as they absorb EB stain. 1×10^5 cells of MDA MB 231 cell line were seeded in a 6-well plate with 3 mL of DMEM medium and cultured for 24 hours at 5% CO₂ in a humidified incubator. The next day, the grown cells were given an IC₅₀ concentration of the cytotoxicity drug prepared in the growth medium. Three of the wells were kept as a control. These cells were then cultured for 24 hours at 5% CO₂ in a humidified incubator. AO and EB stock solutions were produced in PBS solution at a concentration of 10 mg/mL each. To prepare the working solution, 1 μ L of stock solution was diluted with 100 μ L of PBS, resulting in a final concentration of 1 mg/mL for both AO and EB. The AO/EB stain is obtained by combining both stains in a 1:1 ratio. After incubation, 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) was added to the wells and left for 20 minutes. The cells were then viewed using an inverted fluorescence microscope.

4.2.5 DPPH Assay

To evaluate the antioxidant effect, the free radical scavenging activities of Nysatin was determined by DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging method. DPPH is a stable free radical compound that accepts an electron or hydrogen atom to become a stable diamagnetic molecule. When an antioxidant compound is added to a DPPH solution, it can donate an electron or hydrogen atom to the DPPH radical, neutralizing its free radical nature. DPPH assay is based on the colour change of the DPPH radical from purple to yellow as it is reduced by an antioxidant compound. The degree of colour change is directly proportional to the antioxidant activity of the compound being tested. DPPH stock solution (1 mM) was prepared by dissolving 19.715 mg of DPPH in ethanol for 50 mL and the working solution of DPPH (150 μ M) was prepared by dissolving 7.5ml from the stock in 42.5ml of ethanol and stored in dark. Nystatin (3 μ L, 6 μ L, 12 μ L, 24 μ L, 48 μ L, 96 μ L, 100 μ L) were added in duplicates in a 96-well plate with standard control ascorbic acid (3 μ L, 6 μ L, 12 μ L, 24 μ L, 48 μ L, 96 μ L, 100 μ L). Then 100 μ L of DPPH solution was added and incubated for 30 minutes at room temperature in the dark. After incubating for 30 minutes, the absorbance was measured at 517 nm using a multiwell microplate reader and the percentage of inhibition of DPPH radical or the scavenging activity was calculated using the formula.

$$\text{Radical Scavenging Activity \%} = 100 - \left[\left(\frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \right) \times 100 \right]$$

4.2.6 Total RNA Isolation

The cancer cells were treated with an IC₅₀ concentration of the cytotoxicity drug. It was kept in a 5% CO₂ incubator for 24 hours. The cells were rinsed the following day with an ice-cold PBS solution. 1 mL of Trizol is added to the suspended cells, then scrapped. To the trizol cell lysate, 250 μ L of chloroform was added, stirred, and kept at room temperature for 5 minutes. The solution was then centrifuged at 10,000 RPM for 5 minutes. The aqueous phase was then pipetted out without disturbing the contents, transferred to a new 1.5 mL tube, and stored on ice.

Then, 550 μL of isopropanol was added to the aqueous phase and centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellet was recovered and cleaned twice with sterile nuclease-free water and 75% ethanol. The dried pellet was then resuspended in 25 μL of sterile, nuclease-free water. The RNA was then diluted 1:40 with sterile nuclease-free water. To determine the RNA content, the solution was read at 260 and 280 nm using a microplate reader to calculate the $A_{260}:A_{280}$ ratio the amount of RNA per μL was also taken. The RNA was then kept at -20°C until used.

4.2.7 Agarose Gel Electrophoresis

To evaluate RNA integrity, agarose gel electrophoresis was conducted. Initially, a 1% agarose gel was prepared by dissolving agarose in 20 mL of 1x Tris/Boric acid/EDTA (TBE) buffer and heating until complete dissolution. Ethidium Bromide (EtBr) was then added to the solution, followed by pouring into a gel tray with a comb inserted. After solidification for 15 to 30 minutes, the gel tray was immersed in a gel tank filled with 1x TBE buffer (250 mL). Subsequently, 10 μL of total RNA mixed with 6 μL of 6x gel loading dye was loaded onto the gel and electrophoresed at 100 V for 30 minutes. Gel visualisation and documentation were performed using the Gel Doc XR+ Gel Documentation System (Biorad, USA) under UV absorbance. This process facilitated the assessment of RNA integrity, crucial for various molecular biology applications.

4.2.8 cDNA Conversion

100 ng of total RNA was converted to cDNA, using iScript™ cDNA Synthesis Kit according to the manufacturer's protocol in a two-step procedure. 15 μL of total RNA was mixed with 4 μL of the master mix and 1 μL of Reverse Transcriptase (RT) enzyme and was made up to 20 μL volume according to the protocol. The tubes were incubated in a thermal cycler at 25°C for 5 min for priming, 46°C for activating RT enzyme, then at 95°C for 1 min to inactivate reverse transcriptase and were held at 4°C. The tubes were stored at -20°C for further use.

4.2.9 Designing of Real-Time quantitative Polymerase Chain Reaction Primers

The primers for target genes (NF-B, IL 6, TNF, IL 1, BCL 2, BAX) were developed using the Eurofins genomics primer designing program and were given for synthesising to Barcode Biosciences, Bangalore . The ideal conditions for this primer were selected, as follows: primer length: 18 to 20 bp; GC content: 40% to 55%; melting temperature (T_m): 65 to 72 °C; annealing temperature (T_a): 50 to 57 °C. The primers were dissolved in sterile nuclease-free water and diluted to a working concentration of 1 µM for further use.

Table 4.2 List of Primers used for qRT-PCR analysis

S.NO	TARGET GENE	FORWARD PRIMER	REVERSE PRIMER	AMPLICON LENGTH (bp)	T_a (°C)
1	NF-κB	CGAATGGACAA GACAGCAG	CCGGAACACAA TGGCATAC	177	53.7
2	IL 6	ATAACCAACCCC TGACCCCAAC	ATACCTCAAAC TCCAAAAGACC	149	55.1
3	TNF α	AACCATTCTCC TTCTCCCC	TTTCACCTCCAT CCATCCTC	194	54.3
4	IL 1β	CTCTCACCTCT CCTACTCAC	ACACTGCTACT TCTTGCCC	186	51.5
5	BCL 2	CAAACAAGCTC CCCTGAAAC	GCAAAGCCATA GCCTGATAC	205	53.1
6	BAX	TCAGGACACAG GACTCTCAG	ACAAGGGAAGG GGACAAGAC	183	55.8
7	GADPH	GAAGGTCGGAG TCAACGGATTT	CTGGAAGATGC TGATGGGATT	183	56.1



Figure 4.7 Forward and Reverse Primers

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

qRT-PCR was performed with gene-specific primers for four genes (NF- κ B, IL 6, TNF α , IL 1 β) using SensiFASTTM SYBR[®] Lo- ROX Kit (Bioline, UK) according to the manufacturer's protocol in duplicates. 5 μ L of SYBR Green master mix, 1 μ L of forward primer (3 μ M), 1 μ L of reverse primer (3 μ M), 3 μ L of cDNA template (50 ng) was added and the volume was made to 10 μ L with nuclease-free water. PCR amplification was performed with the Mastercycler Nexus Gradient Thermal Cycler (Eppendorf, Germany). The thermal-cycling conditions included 95°C for 2 min (polymerase activation), 95°C for 5 sec, 60 to 65°C for 10 sec, 72°C for 15 sec for 40 cycles. The program was set up to calculate Cycle threshold value (C_t value) for 40 cycles. The relative expression levels between target and control were measured with the Relative quantification ($2^{-\Delta\Delta C_t}$) method. In this method, the relative gene expression is assumed to be 1 for the reference sample. It assumes 100% efficiency and uniform PCR amplification across all the samples. The results were reported as expression fold change when compared to control. Fold change >1.0 was taken as upregulation and < 1.0 was considered as downregulation in the gene expression.

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

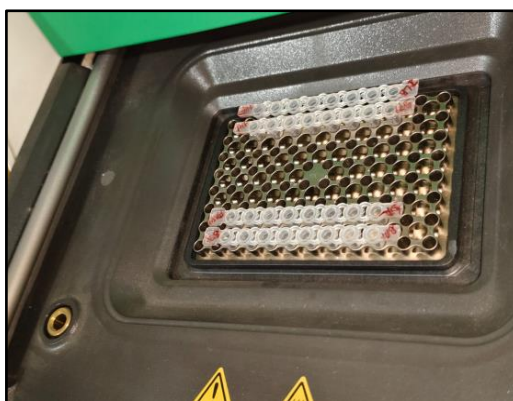


Figure 4.8 Thermocycler

4.3 STATISTICAL ANALYSIS

GraphPad Prism is a strong statistical analysis and graphing programme that is commonly used in scientific research. It's simple interface facilitates data visualisation, curve fitting, and statistical analysis. Prism, with features like nonlinear regression and automated error bar creation, simplifies data analysis, making it a popular option among researchers. The tests that were performed are detailed in the table below.

Table 4.3 List of tests used for Statistical Analysis

S.NO	EXPERIMENT	STATISTICAL TEST	PURPOSE
1	MTT Assay	Unpaired T-Test	Compare the effect of the drug on normal and cancer cells.
2	AO/EB Staining	Chi-square Test	To assess whether the drug treatment has a significant effect on the occurrence of apoptosis in cancer cells.
3	qRT-PCR	Unpaired T Test	To compare gene expression levels between different treatment groups (untreated vs. drug-treated) in cancer cells and interpret the results effectively.

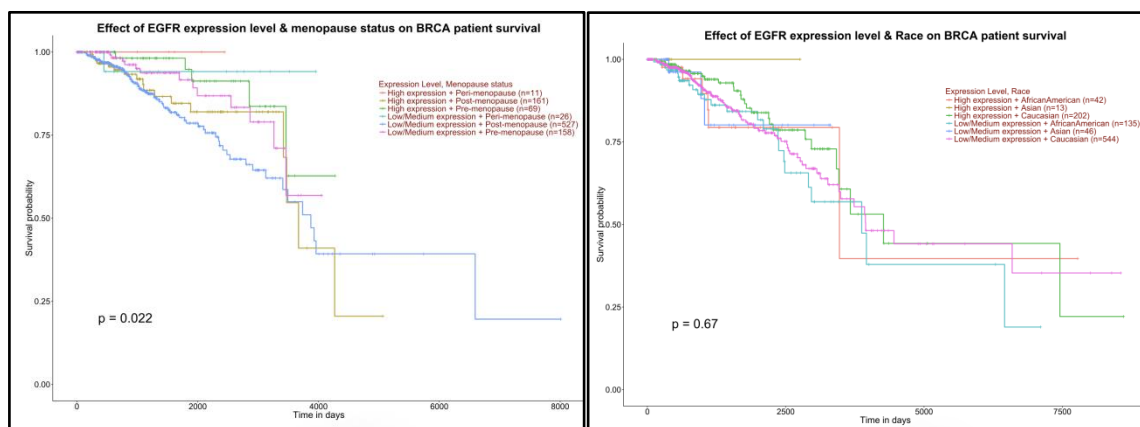
CHAPTER 5

RESULTS AND DISCUSSION

5.1 IN SILICO STUDIES

5.1.1 Pan-cancer survival analysis of EGFR using TCGA

As analysed from the graphs, the probability of survival increases over time for all expression levels of EGFR, indicating that EGFR is a moderate prognosis factor for Breast cancer. Patients expressing higher levels of EGFR were observed to survive longer than patients with high expression levels and hence can be considered as a suitable target for treating TNBC. According to the effect of EGFR expression and menopause status on BRCA patient survival graph, the patients with low/medium expression and post-menopause have higher 0.25 survival probability with longer number of days. It also shows that the patients with low/medium expression of EGFR and gender as male have higher rate of survival and can live a longer number of days. It also shows that caucasians (North Americans) have a higher survival rate. Based on the subtype, luminal subtype with low/ moderate expression have a longer number of survival days. From these considered factors, it is evident that low/moderate expression of EGFR constitutes a higher survival rate and can be used as a suitable target for treating BC.



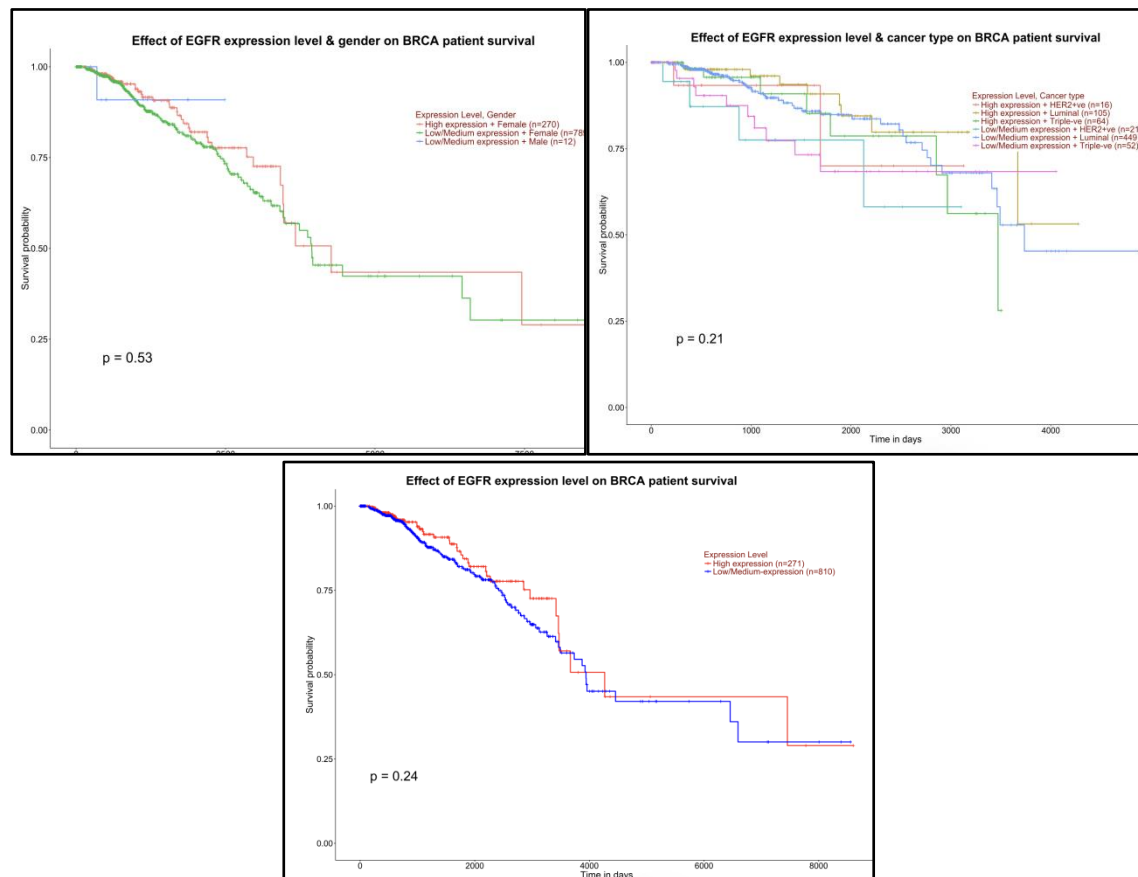


Figure 5.1 Survival Analysis of EGFR using UALCAN

5.1.2 Pan-cancer survival analysis of EGFR using KM

The Kaplan-Meier plot illustrates the event-free survival probabilities over time for two cohorts categorised by their EGFR expression levels. Notably, the survival curve for the low EGFR group consistently exceeds that of the high EGFR group, indicating a more favourable prognosis for patients with lower EGFR expression. Supported by a hazard ratio of less than 1 and a significant logrank p-value of 0.00023, the statistical comparison confirms the observed disparity in survival outcomes between the two groups. With a larger initial population in the low EGFR group and substantially fewer events occurring over time compared to the high EGFR group, these findings underscore the potential prognostic relevance of EGFR expression levels in the context of survival outcomes. It is evident from the graph, Lower EGFR expression correlates with better survival outcomes, supported by significant statistical findings in Kaplan-Meier analysis.

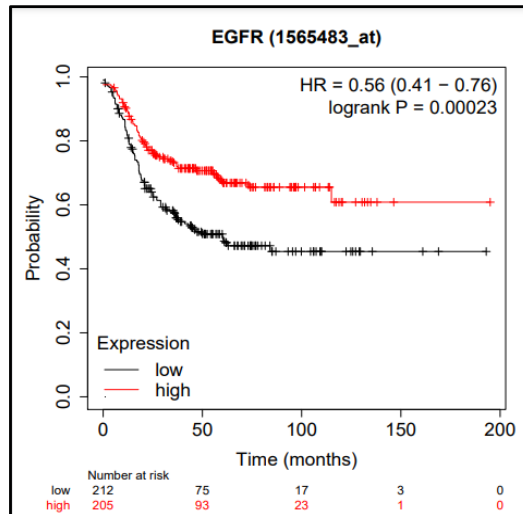


Figure 5.2 Survival Analysis of EGFR using KM plotter

5.1.3 Validation of EGFR Survival Analysis Study using GEPIA

The plot showcases survival curves for patients categorised by high and low EGFR TPM levels, presenting their event-free survival probabilities over time. Notably, both curves exhibit similar trends, indicating comparable survival outcomes between the two groups. The hazard ratio of 1.1 suggests a slight disadvantage for the high EGFR TPM group, albeit not significantly different from the low EGFR TPM group. This marginal difference in prognosis is further underscored by the logrank p-value of 0.64, signifying a lack of statistical significance in survival disparities between the cohorts. Initial patient counts at the study's onset are equal between the high and low EGFR TPM groups, and the number of events—presumably deaths—over time is also similar, further supporting the conclusion of comparable survival outcomes. Overall, the Kaplan-Meier analysis implies that EGFR TPM levels do not distinctly influence survival prognosis, as evidenced by the absence of statistically significant differences in survival curves or hazard ratios. This graph validates the above two survival graphs visually that low EGFR TPM correlates to better survival outcome and that EGFR is a moderate prognosis factor that can be used as a potential target for treating Breast cancer.

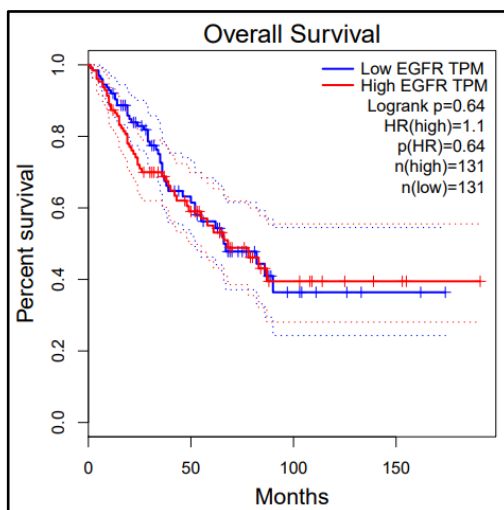


Figure 5.3 Survival Analysis of EGFR using GEPIA

5.1.4 In Silico Characterization of Nystatin

a) General Characteristics

Nystatin is a potent antifungal drug that has a complicated chemical structure made up of many conjugated double bonds and a polyene with a macrolide ring. Its enormous, complex character is highlighted by its molecular weight of 926.1 g/mol and chemical formula, C₄₇H₇₅NO₁₇. By attaching itself to ergosterol, a crucial part of fungal cell membranes, it causes damage to the membranes' integrity and ultimately causes cell death. Because of this special quality, nystatin can successfully treat a variety of fungal diseases, especially candidiasis. Since it only targets fungal cells, the toxicity to human cells is reduced, which makes it an essential drug for treating a variety of fungal conditions.

b) Lipophilicity

The table below pertains to the octanol/water partition coefficient (LogP), a critical parameter in drug distribution within the body. The LogP values include iLOGP, XLOGP3, WLOGP, MLOGP, SILICOS-IT, and CONSENSUS LOG POW. iLOGP (-4.30) signifies strong hydrophilicity, indicating a pronounced affinity for water over fats and oils. XLOGP3 (-0.20) indicates moderate hydrophilicity with some

lipophilic traits, suggesting a balanced affinity for both water and non-polar solvents. Conversely, WLOGP (0.94) denotes moderate lipophilicity, indicating a higher preference for non-polar solvents. MLOGP (-1.67) suggests hydrophilicity with slight lipophilic characteristics, prioritising water over non-polar solvents. SILICOS-IT (-4.26) aligns closely with iLOGP, indicating strong hydrophilicity. CONSENSUS LOG P_{OW} (-0.18) reflects a collective assessment, indicating moderate hydrophilicity with weak lipophilic tendencies.

In summary, the drug exhibits varying degrees of hydrophilicity and lipophilicity, facilitating its solubility in both polar and non-polar environments. While there may be slight discrepancies among different calculation methods, the consensus suggests a predominantly hydrophilic nature with some lipophilic properties.

Table 5.1 Lipophilicity

iLOGP	XLOGP3	WLOGP	MLOGP	SILICOS-IT	CONSENSUS LOG P_{OW}
4.30	-0.20	0.94	-1.67	-4.26	-0.18

c) Water solubility

The drug's water solubility is assessed by multiple methods, yielding different predictions. According to the ESOL model, it is moderately soluble (-5.26 Log S), indicating limited but not highly insoluble characteristics. Conversely, the Ali method suggests poor solubility (-6.06 Log S), with only a small amount dissolving in water. In contrast, the SILICOS-IT method predicts high solubility (2.54 Log S), indicating excellent water solubility with a large quantity dissolving in water. Solubility values range from 5.12e-03 mg/ml to 3.20e+05 mg/ml, reflecting varying degrees of solubility across methods. These methods suggest that Nystatin is moderately soluble in water and exhibits partial lipophilic characteristics.

Table 5.2 Water Solubility

	ALI	
1	Log S	-4.78
2	Solubility	7.99e- 03 mg/ mL 1.66e-05 mol/L
3	Class	Moderately soluble
	SILICOS IT	
1	Log S	-4.50
2	Solubility	1.53e-02 mg/mL 3.16e-05 mol/L
3	Class	Moderately soluble
	ESOL	
1	Log S	-4.14
2	Solubility	3.46e-02 mg/mL 7.17e-05 mol/mL
3	Class	Moderately soluble

d) Pharmacokinetic Parameters

The table describes the pharmacokinetic properties of Nystatin. It exhibits low gastrointestinal absorption, indicating inefficient uptake into the bloodstream following oral administration. Additionally, it does not permeate the Blood-Brain Barrier (BBB), suggesting limited passage into the central nervous system. As a substrate for P-glycoprotein (P-gp), its absorption and distribution are subject to active transport mechanisms mediated by P-gp. However, the drug does not inhibit crucial cytochrome P450 (CYP) enzymes responsible for drug metabolism, minimising potential interactions with other medications. Furthermore, its Log Kp

value of -12.09 cm/s indicates minimal skin permeation, rendering transdermal absorption unlikely. Nystatin exhibits low gastrointestinal absorption and negligible Blood-Brain Barrier penetration. It's a P-glycoprotein substrate, unaffected by CYP enzyme inhibition, with minimal skin permeation. These traits define its pharmacokinetic profile.

Table 5.3 Pharmacokinetics Parameters

GI ABSORPTION	LOW
BBB PERMEANT	NO
P-GP SUBSTRATE	YES
CYP1A2	NO
CYP2C19	NO
CYP2C9	NO
CYP2D6	NO
CYP3A4	NO
LOG KP (cm/s)	-12.09 cm/s

e) Drug-likeness

Lipinski's Rule of Five (Ro5) evaluates drug-likeness, where violating multiple criteria indicates reduced oral bioavailability. Ghose's filter assesses drug-likeness through varied parameters, flagging deviations from the filter's optimal range. Veber's rule evaluates oral bioavailability, with a Total Polar Surface Area (TPSA) over 140 as a violation. Egan's rule sets a TPSA threshold of 131.6 for oral bioavailability assessment. Muegge's rule imposes stricter criteria, challenging compounds violating it in terms of bioavailability and drug-likeness. A bioavailability score of 0.17 suggests moderate bioavailability based on specified

criteria. Guidelines like Lipinski's Rule, Ghose's filter, and bioavailability score indicate potential challenges in drug-likeness and oral bioavailability.

Table 5.4 Drug-likeness

LIPINSKI	No; 3 violations: MW>500, NorO>10, NHorOH>5
GHOSE	No; 3 violations: MW>480, MR>130, #atoms>70
VEBER	No; 1 violation: TPSA>140
EGAN	No; 1 violation: TPSA>131.6
MUEGGE	No; 4 violations: MW>600, TPSA>150, H-acc>10, H-don>5
BIOAVAILABILITY SCORE	0.17

f) Medicinal Chemistry Properties

The analysis of the medicinal chemistry properties of Nystatin, it is evident that the drug does not contain problematic substructures responsible for false-positive results. The compound does not contain any of the flagged structures. The drug is said to violate the Lead Likeness parameter, suggesting that the compound's molecular weight exceeds the recommended threshold of 350, indicating that it might be heavier compared to that of the typical lead-like compounds. Synthetic accessibility is a measure of how easy it is to synthesise a compound. A score of 10.00 suggests that the compound is deemed to be synthetically accessible, meaning it can be feasibly synthesised using common chemical methods.

Nystatin does not trigger any problematic alerts in terms of common interference compounds (PAINS alerts, Brenk alerts) and shows good synthetic accessibility. However, it violates the Lead Likeness rule due to its higher molecular weight, suggesting that it might be outside the typical range for lead compounds.

Table 5.5 Medicinal Chemistry Properties

PAINS	BRENK	LEADLIKENESS	SYNTHETIC ACCESSIBILITY
0 alert	0 alert	No; 1 violation: MW>350	10.00

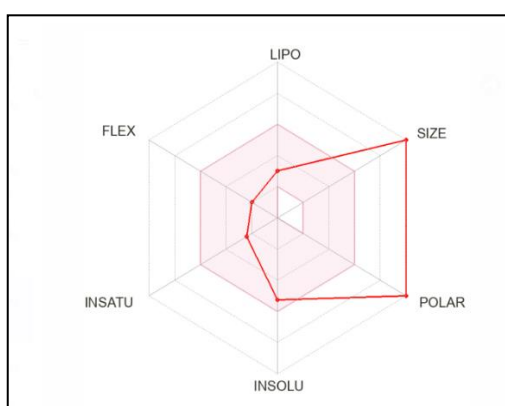
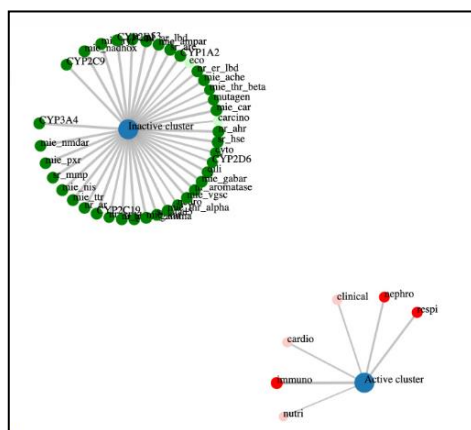


Figure 5.4 Various properties of Nystatin

g) Toxicity

The table outlines predictions for various toxicity endpoints, such as hepatotoxicity and immunotoxicity, along with associated probabilities. Each prediction ("Active" or "Inactive") is accompanied by a probability score, reflecting the model's confidence level in its prediction. For instance, hepatotoxicity is predicted as "Inactive" with a probability of 0.97, indicating high confidence. These assessments are generated by a model based on input data and training, providing insights into the likelihood of toxicity endpoints being active or inactive. Higher probabilities signify greater confidence in the model's predictions. The model predicts toxicity endpoints such as hepatotoxicity and immunotoxicity, with high confidence levels based on associated probabilities. The data predicts that the drug belongs to the Class 6 toxicity with LD50 as 8000mg/kg of body mass.

<i>TARGET</i>	<i>HP</i>	<i>CG</i>	<i>IT</i>	<i>MG</i>	<i>CT</i>
PREDICTION	NO	NO	YES	NO	NO
PROBABILITY	0.97	0.73	0.99	0.89	0.83



5.1.5 Molecular Docking using PyRx and Validation by iGEMDock

43

indicating that the docking is relatively stable. Consequently, Nystatin shows promise for further exploration as a potential therapy targeting EGFR. These results support its candidacy for targeted therapy against EGFR, emphasising its potential in drug development and molecular targeting approaches.

Table 5.7 Binding scores for the docked molecule

BINDING SCORE USING PYRX	BINDING SCORE USING iGEMDOCK
-8.3 kcal/mol	-94.924 kcal/mol

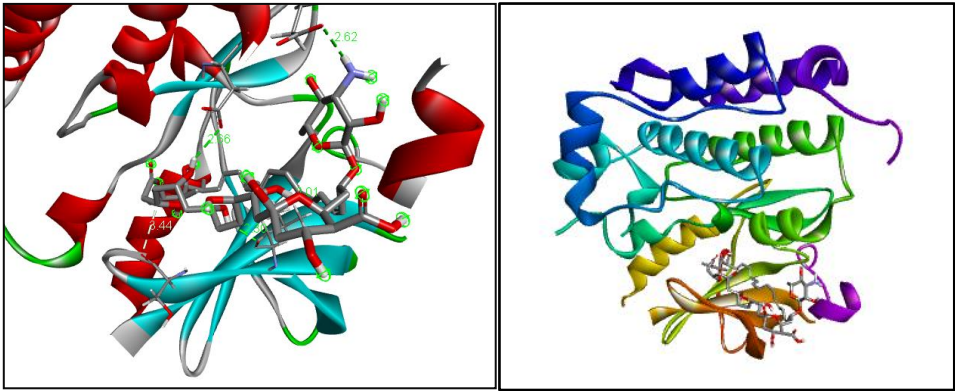


Figure 5.6 3D Structure of the Docked molecules

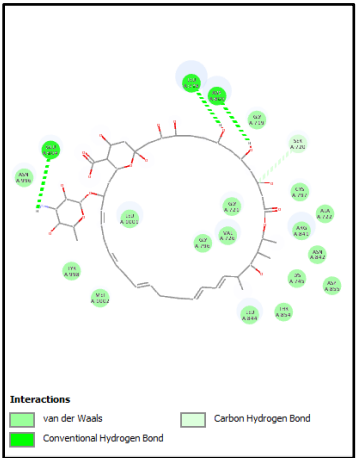


Figure 5.7 2D Structure of the Docked molecules

5.2 IN VITRO STUDIES

5.2.1 MTT Cytotoxicity Assay

The Cytotoxicity assay for the two cell lines, Hela and MDA MB 231 was performed using MTT dye. The cells were treated with different concentrations of Nystatin for 24 hours. Compared to the Hela cells, the MDA MB 231 cells showed a higher percentage of cytotoxicity at different concentrations. The results suggest that there is a higher sensitivity against Nystatin for the MDA MB 231, compared to that of the normal Hela cells. Unpaired T test was used to prove the statistical difference between the normal and the cancer cells with significance rate at 95% or 0.05. The study investigated the effects of varying drug concentrations on both normal and cancer cells, comparing within-group differences and between-group disparities. For normal cells, the analysis revealed a significant contrast between the effect of the drug at 0 $\mu\text{g}/\text{ul}$ concentration and other concentrations ($p = 0.0066$), leading to the rejection of the null hypothesis (H_0) in favour of the alternative hypothesis (H_1). Similarly, in cancer cells, the comparison within groups demonstrated a notable difference ($p = 0.0075$), supporting the rejection of H_0 . Furthermore, between-group analysis indicated a significant discrepancy in the drug's effect on normal cells versus cancer cells ($p = 0.0132$), supporting the alternative hypothesis. These findings collectively suggest that Nystatin has a statistically significant impact on both normal and cancer cells, with differences observed both within and between these cell types.

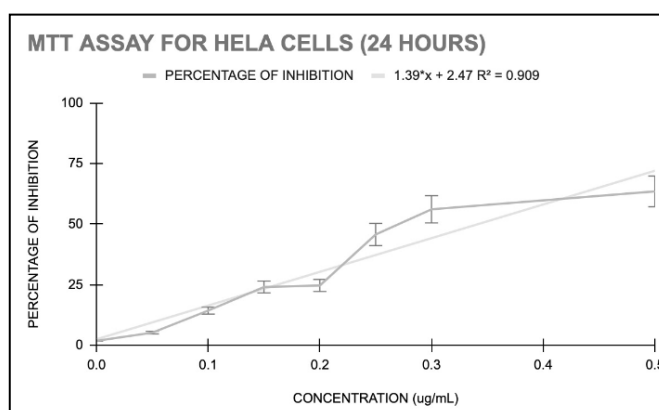


Figure 5.8 Inhibition plot for Normal Cells

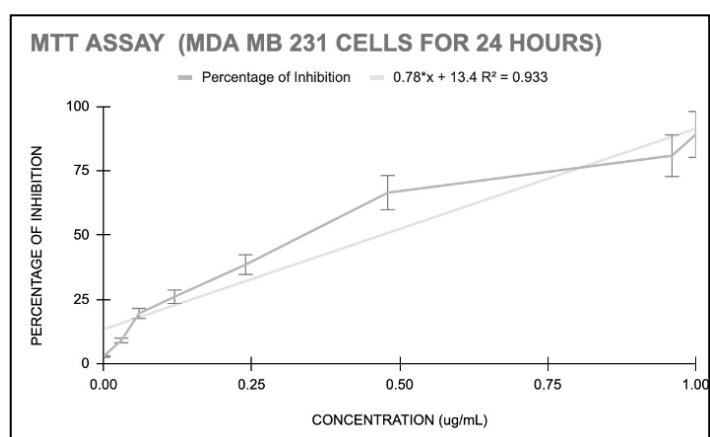


Figure 5.9 Inhibition plot for Cancer cells

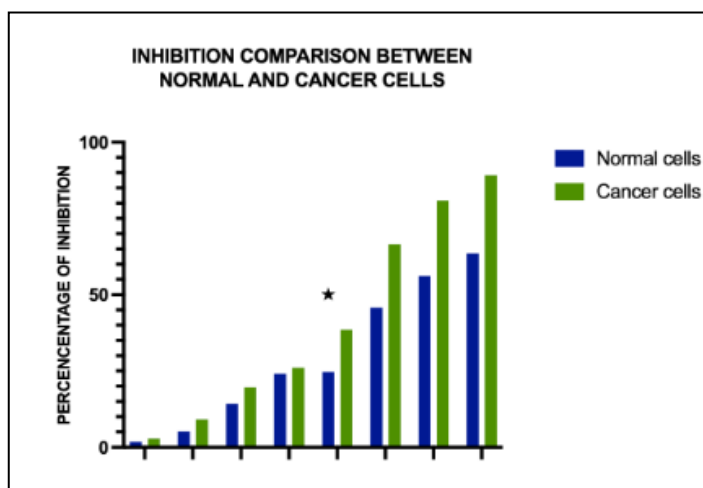


Figure 5.10 Inhibition Comparison between Normal and Cancer cells; The p -value of the above mentioned test is calculated to be 0.00132.

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

The comparative analysis of treated cells (Figure 5.12) and control cells (Figure 5.11) reveals a pronounced disparity in Ethidium Bromide (EB) concentration, indicating a higher permeability of damaged membranes in the treated group. EB selectively enters cells with compromised membranes, staining their nuclei red, while Acridine Orange (AO) stains all nuclei green. In the treated cells, the dominance of EB over AO results in a predominantly red appearance of nuclei,

signalling membrane damage and potentially increased apoptosis rates. This inference is supported by the graph (Figure 5.13), which illustrates a greater count of cells in late apoptosis in the treated group compared to the control. Early apoptosis, characterised by green nuclei with chromatin condensation, transitions to late apoptosis where DNA fragmentation becomes apparent, manifesting as orange or red nuclei. Necrotic cells, identifiable by swollen and damaged membranes, also stain red with EB. The observed higher concentration of EB in treated cells, coupled with the graph's depiction of increased late apoptosis, suggests a greater propensity for apoptosis induction in the treated group. These findings collectively imply a significant impact of the Nystatin treated group on cellular viability, potentially through mechanisms involving membrane integrity and apoptosis induction. A Chi-square test was done to validate the above statement statistically at a significance level of 95% ($\alpha = 0.05$) with 2 degrees of freedom, tested the null hypothesis (H_0) stating no difference in proportions of live, dead, and apoptotic cells between control and treated groups, against the alternative hypothesis (H_1) suggesting a significant difference. The obtained p-value of $p < 0.0001$ led to the rejection of H_0 in favour of H_1 . Thus, there exists a statistically significant difference in the proportions of live, dead, and apoptotic cells between the control and treated groups, implying a notable impact of the treatment on cellular outcomes.



Figure 5.11 Control (Acridine Orange); Control (Ethidium Bromide); Control (Acridine Orange + Ethidium Bromide)

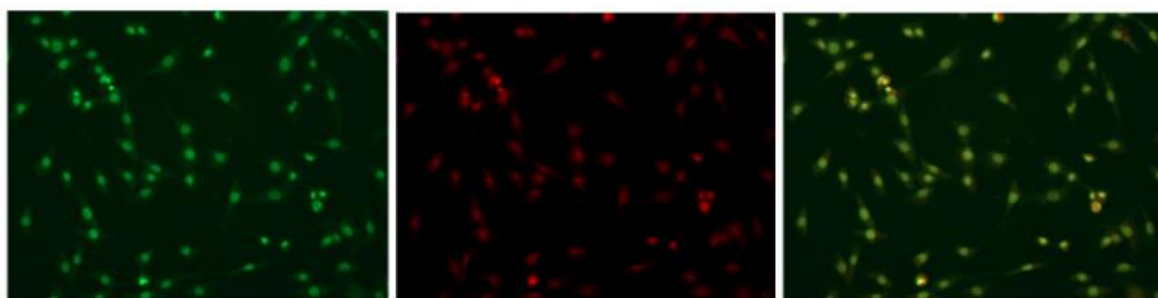


Figure 5.12 Treated (Acridine Orange); Treated (Ethidium Bromide); Treated (Acridine Orange + Ethidium Bromide)

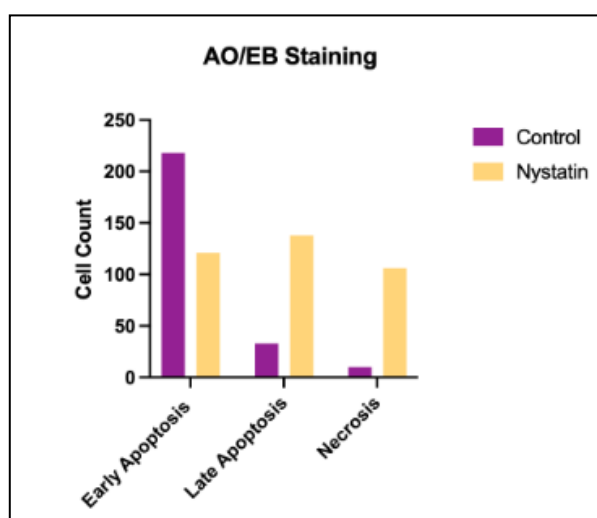


Figure 5.13 Graphical Representation of AO/EB Staining results; The p-value of the above mentioned test is calculated to be $p < 0.0001$.

5.2.3 DPPH Assay

In this assay, the IC₅₀ values of nystatin and ascorbic acid (used as a standard) were determined as 5.29 µg/ml and 15.29 µg/ml, respectively. The IC₅₀ value represents the concentration at which 50% of the free radicals (DPPH) are neutralized by the compound being tested. A lower IC₅₀ value indicates greater antioxidant potency. The significantly lower IC₅₀ value of nystatin compared to ascorbic acid suggests that nystatin possesses stronger antioxidant activity against DPPH radicals. This finding underscores the potential of nystatin as an

effective antioxidant agent. Antioxidants play a crucial role in neutralizing harmful free radicals, thereby mitigating oxidative stress-induced damage in cancer.

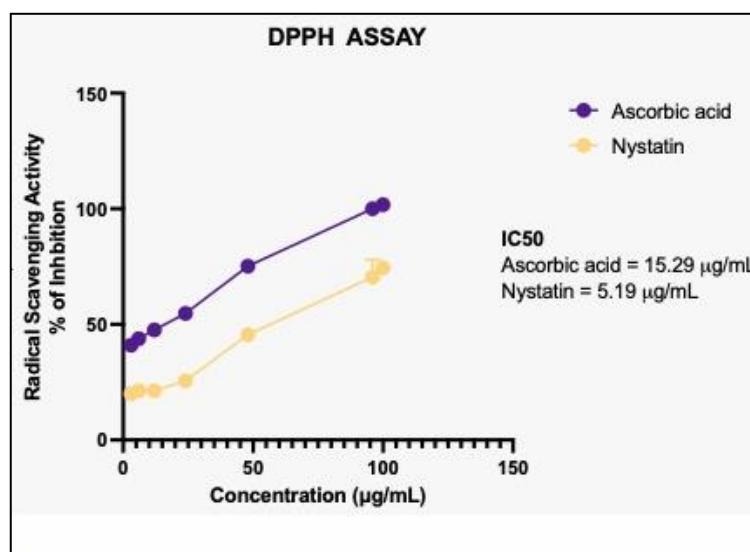


Figure 5.14 DPPH Assay

5.2.4 RNA Isolation and cDNA conversion

The RNA isolated from drug-treated MDA MB 231 cells formed clear white pellets post-precipitation and centrifugation with isopropanol. These pellets were air dried and resuspended in 20 µL sterile water. Subsequently, the RNA sample underwent preparation for Agarose Gel Electrophoresis, with UV exposure in a dark room facilitated by a Biorad UV exposure unit (USA). The resulting agar gel exhibited distinct bands, with larger molecular weight bands identified as mRNA and smaller ones as t-RNA. This characteristic banding pattern confirmed the integrity of the RNA samples. Following visualisation, quantification was performed using a microplate reader, and the RNA amount was determined via the $A_{260}:A_{280}$ ratio. This multi-step process ensures the purity, integrity, and quantification of the isolated RNA, essential for downstream molecular biology applications.

The RNA underwent preparation for cDNA conversion by calculating the necessary dilution to match the total conversion volume capacity of the cDNA conversion kit. The sample RNA was diluted using nuclease-free water.

Subsequently, the RNA sample was combined with the provided master mix and run on a thermocycler to obtain the cDNA.

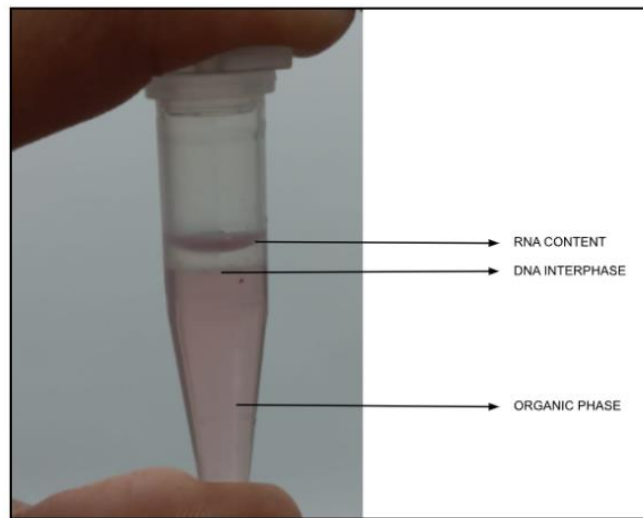


Figure 5.15 Phase Separation during RNA Isolation



Figure 5.16 cDNA conversion

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

The qPCR was done for four inflammatory genes (TNF α , IL 6, IL 1 β , NF κ B) and two apoptotic genes (BCL 2, BAX) using GAPDH as the housekeeping gene. The results were obtained in the relative quantification mode and the cycle threshold C_t value was obtained. The fold change was calculated using two groups: Control group without Nystatin treatment. This group shows the expression of the gene

after the diseased model is treated with the Nystatin. By normalising the expressions and comparing it with the control group, the change in expression can be analysed. It assumes that PCR amplification is 100% efficient and consistent across all samples. The results were presented as expression fold change as compared to the control. Gene expression was classified as upregulated if the fold change was greater than 1.0 or downregulated if it was less than 1.0. As from the graphs, it is apparent that all genes have been downregulated except for BAX. BAX is a pro-apoptotic protein whose expression must be higher to perform apoptosis. As expected the expression of BAX was slightly upregulated. Statistical test was done to validate the below results.

Table 5.8 Fold Change for various genes (TNF α , IL 6, IL 1 β , NF κ B, BCL 2, BAX)

S.NO	SAMPLE	RELATIVE QUANTIFICATION (FOLD CHANGE)
1	Control	1.000000000
2	TNF α	0.1303048331
3	IL 6	0.7553624787
4	IL 1 β	0.5612427296
5	NF κ B	0.109974561
6	BCL 2	0.1460442075
7	BAX	1.420835859

Using an unpaired T-test with a significance level of 0.05 (95% confidence), the study evaluated gene expression differences between control (untreated) and treated (drug-treated) cells. For TNF α , NF κ B, and BCL 2 genes, p-values were 0.0025, 0.0015, and 0.0019, respectively, indicating a statistically significant difference, leading to the rejection of the null hypothesis (H_0) and acceptance of the alternative hypothesis (H_1). Conversely, for IL 6, IL 1 β , and BAX genes, p-

values were 0.4116, 0.1616, and 0.6566, respectively, suggesting no significant difference, hence, accepting H_0 . Thus, the study concludes a significant alteration in TNF A, NFKB, and BCL 2 gene expressions post-drug treatment, while expressions of IL 6, IL 1B, and BAX remain unaffected. These findings elucidate the molecular effects of drug treatment on gene expression profiles in the studied cells.

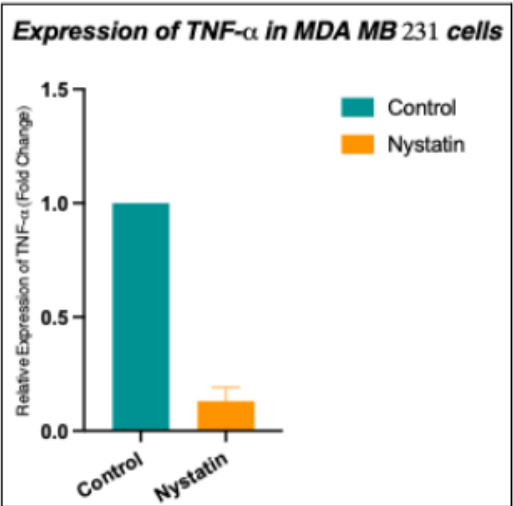


Figure 5.17 Expression of TNF α

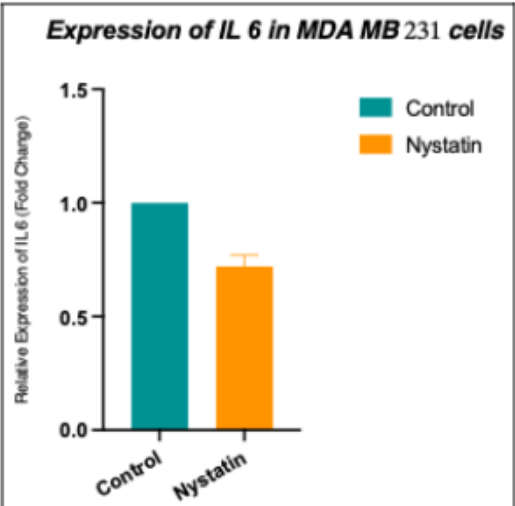


Figure 5.18 Expression of IL 6

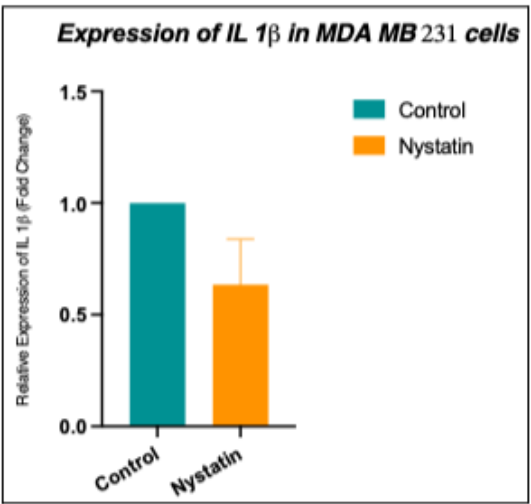


Figure 5.19 Expression of IL 1 β

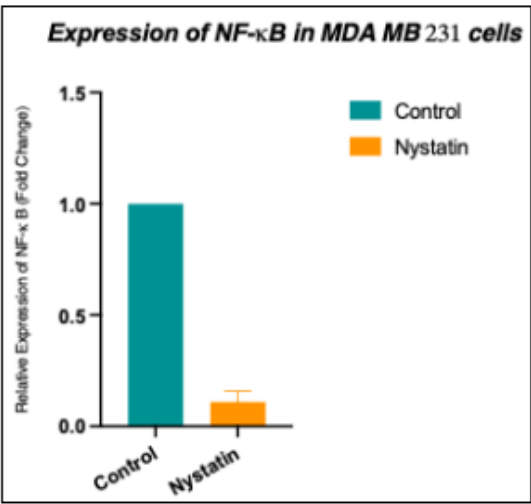


Figure 5.20 Expression of NF κ B

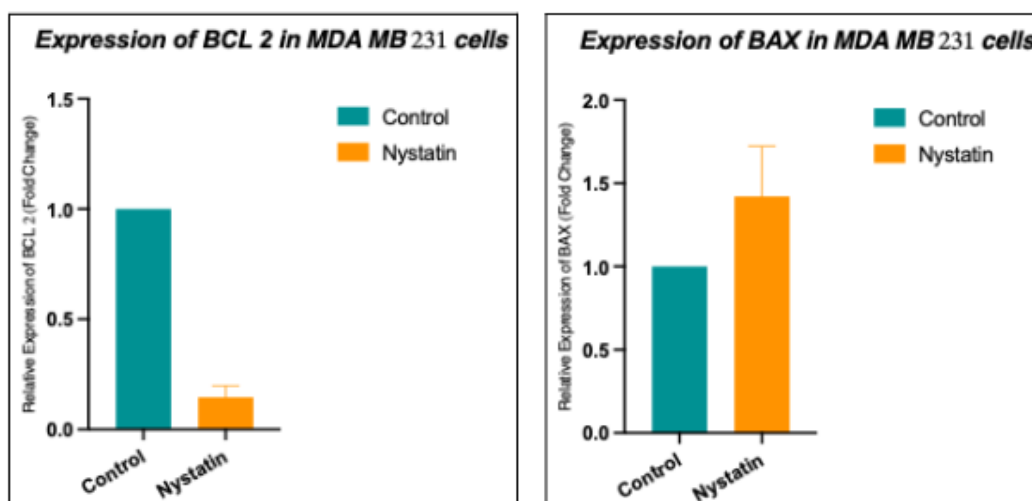


Figure 5.21 Expression of BCL 2 **Figure 5.22 Expression of BAX**

The present study aimed to explore the potential therapeutic efficacy of Nystatin in breast cancer by elucidating its molecular interactions, evaluating its cytotoxic effects on cancer cells, and analyzing its impact on gene expression associated with NF κ B signaling and cytokine production. Through a multi-faceted approach encompassing *In Silico*, *In Vitro*, and molecular biology techniques, the study sought to provide comprehensive insights into the pharmacological profile and therapeutic potential of Nystatin in breast cancer treatment which certainly aligns with the student outcome to design an experimental procedure.

Molecular docking studies were conducted to elucidate the possible binding interactions between Nystatin and EGFR, a pivotal molecular target implicated in breast cancer progression. The results revealed a robust interaction between Nystatin and EGFR, as evidenced by a significant negative binding affinity, indicating a high likelihood of stable binding. Moreover, the precise alignment between the molecules, as indicated by low Root Mean Square Deviation (RMSD) values, underscored the efficacy of the binding interaction. These findings suggest that Nystatin may exert its therapeutic effects, at least in part, through inhibition of EGFR-mediated signaling pathways implicated in breast cancer pathogenesis and coordinates with program outcome to use modern tools for prediction and screening of drugs which almost complies with F. F. Sherbiny *et al.*, 2021.

To assess the cytotoxic effects of Nystatin on breast cancer cells, MTT assays were performed, revealing a concentration-dependent decrease in cell viability following Nystatin treatment. Interestingly, breast cancer cells exhibited higher sensitivity to Nystatin compared to normal cells, highlighting its potential as a selective cytotoxic agent against cancer cells. Statistical analyses further corroborated these findings, demonstrating significant differences in cytotoxicity between Nystatin-treated cancer cells and untreated controls. These results suggest that Nystatin exerts potent anti-proliferative effects on breast cancer cells, potentially by inducing apoptosis or inhibiting cell proliferation pathways [S. Ranjitkar *et al.*, 2021]. The dual Acridine Orange/Ethidium Bromide (AO/EB) staining revealed Nystatin-induced cellular damage in breast cancer cells, characterized by compromised membrane integrity and increased permeability. Nystatin-treated cells exhibited a higher proportion of late apoptotic cells, supported by chromatin condensation and DNA fragmentation. The AO/EB staining assay provides direct visual evidence of Nystatin-induced cellular alterations, complementing other experimental assays and enhancing our understanding of its mechanism of action in breast cancer cells [Liu, K. *et al.*, 2015]. The DPPH assay demonstrated Nystatin's pronounced antioxidant potency, reflected in its lower IC₅₀ compared to ascorbic acid. This suggests Nystatin's potential therapeutic efficacy extends beyond its antifungal properties. Future investigations should delve into its mechanisms of action and explore clinical applications in TNBC where oxidative stress plays a significant role. These findings highlight nystatin as a promising candidate for antioxidant-based therapies, offering avenues for novel treatment strategies and emphasizing its relevance in combating diseases associated with oxidative damage [Singh, K., *et al.*, 2018].

To elucidate the molecular mechanisms underlying the cytotoxic effects of Nystatin, gene expression analysis was conducted using quantitative polymerase chain reaction (qPCR). The expression levels of genes involved in NFκB signaling and cytokine production were evaluated, shedding light on the impact of Nystatin on key regulatory pathways implicated in breast cancer progression. Notably, Nystatin treatment led to significant downregulation of NFκB expression, a master regulator of inflammation and oncogenesis, suggesting its potential role in

suppressing pro-survival and pro-inflammatory signaling cascades in breast cancer cells. Additionally, alterations in the expression levels of other cytokines and apoptotic markers were observed, further elucidating the complex interplay between Nystatin and intracellular signaling pathways governing cancer cell fate [Brasier, A.R. 2010].

Collectively, the findings from this study provide compelling evidence for the therapeutic potential of Nystatin in breast cancer treatment. By elucidating its molecular interactions, cytotoxic effects, and impact on gene expression profiles with proven statistical analysis which satisfies the student outcome for ability to develop and conduct experiments and test hypotheses, analyse and interpret data and use scientific judgment to draw conclusions. Moreover, the selective cytotoxicity of Nystatin against cancer cells, coupled with its ability to modulate key signaling pathways implicated in breast cancer progression, highlights its promise as a targeted therapeutic agent for breast cancer patients. Further preclinical and clinical investigations are warranted to validate these findings and elucidate the translational potential of Nystatin in the clinical management of breast cancer.

CHAPTER 6

SUMMARY AND CONCLUSION

Breast cancer is a complex disease with various subtypes, posing significant challenges in treatment and management. Among these subtypes, Triple-Negative Breast Cancer (TNBC) stands out for its aggressiveness and lack of targeted treatment options due to the absence of estrogen, progesterone, and HER2 receptors. This subtype often requires innovative therapeutic approaches. Repurposing existing drugs for TNBC treatment has emerged as a promising strategy. One such candidate is Nystatin, traditionally used to treat fungal infections. Recent research has explored its potential in TNBC therapy, particularly in targeting the Epidermal Growth Factor Receptor (EGFR), a protein often overexpressed in TNBC cells. *In Silico* validation, a computational method, has been employed to assess the efficacy of nystatin in TNBC treatment. The results indicate promising outcomes, suggesting that nystatin could efficiently target EGFR and inhibit TNBC cell proliferation. Experimental validation through MTT assays, Acridine Orange/Ethidium Bromide (AO/EB) staining, DPPH assay, and quantitative polymerase chain reaction (qPCR) further support the anticancer properties of nystatin. These experiments reveal its ability to suppress TNBC cell growth and induce apoptosis, as evidenced by decreased expression of pro-inflammatory genes such as Tumour Necrosis Factor alpha (TNF α) and Interleukin 6 (IL-6), along with anti-apoptotic gene Bax. In conclusion, the repurposing of nystatin for TNBC treatment, particularly in targeting EGFR, shows promise in both computational and experimental studies. Its efficacy in inhibiting TNBC cell proliferation and modulating gene expression associated with inflammation and apoptosis underscores its potential as a therapeutic agent. Further research and clinical trials with the implementation of Nanotechnology such as liposomal nanoparticles, metallic nanoparticles and other, help in coming up with a potent drug with more target based precision and increased efficiency and are warranted to fully elucidate the safety and efficacy of Nystatin in TNBC therapy, potentially offering a novel approach to combat this aggressive subtype of breast cancer.

REFERENCES

A Chen, S. C., Slavin, M. A., & Sorrell, T. C. (2005). Echinocandin antifungal drugs. In *Fungal Infections in the Immunocompromised Patient* (pp. 507–530). CRC Press. <http://dx.doi.org/10.1201/b14156-21>

Almeida-Ferreira, C., Marto, C. M., Carmo, C., Almeida-Ferreira, J., Frutuoso, C., Carvalho, M. J., Botelho, M. F., & Laranjo, M. (2024). Efficacy of cold atmospheric plasma vs. chemotherapy in triple-negative breast cancer: A systematic review. *International Journal of Molecular Sciences*, 25(6). <https://doi.org/10.3390/ijms25063254>

Attard, G., Reid, A. H. M., Auchus, R. J., Hughes, B. A., Cassidy, A. M., Thompson, E., Oommen, N. B., Folkerd, E., Dowsett, M., Arlt, W., & de Bono, J. S. (2012). Clinical and biochemical consequences of CYP17A1 inhibition with abiraterone given with and without exogenous glucocorticoids in castrate men with advanced prostate cancer. *The Journal of Clinical Endocrinology & Metabolism*, 97(2), 507–516. <https://doi.org/10.1210/jc.2011-2189>

Brasier, A. R. (2010). The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. *Cardiovascular Research*, 86(2), 211–218. <https://doi.org/10.1093/cvr/cvq076>

Britton, K. M., Eyre, R., Harvey, I. J., Stemke-Hale, K., Browell, D., Lennard, T. W. J., & Meeson, A. P. (2012). Breast cancer, side population cells and ABCG2 expression. *Cancer Letters*, 323(1), 97–105. <https://doi.org/10.1016/j.canlet.2012.03.041>

Burguin, A., Diorio, C., & Durocher, F. (2021). Breast cancer treatments: Updates and new challenges. *Journal of Personalized Medicine*, 11(8). <https://doi.org/10.3390/jpm11080808>

Changavi, A. A., Shashikala, A., & Ramji, A. S. (2015a). Epidermal growth factor receptor expression in triple negative and nontriple negative breast carcinomas. *Journal of Laboratory Physicians*, 7(2), 79–83. <https://doi.org/10.4103/0974-2727.163129>

Chen, J., Wei, Y., Yang, W., Huang, Q., Chen, Y., Zeng, K., & Chen, J. (2022a). IL-6: The link between inflammation, immunity and breast cancer. *Frontiers in Oncology*, 12, 903800. <https://doi.org/10.3389/fonc.2022.903800>

Chen, Y., Wang, S., Lu, X., Zhang, H., Fu, Y., & Luo, Y. (2020). Cholesterol sequestration by nystatin enhances the uptake and activity of endostatin in endothelium via regulating distinct endocytic pathways. *Blood*, 117(23), 6392–6403. <https://doi.org/10.1182/blood-2010-12-322867>

Devanaboyina, M., Kaur, J., Whiteley, E., Lin, L., Einloth, K., Morand, S., Stanbery, L., Hamouda, D., & Nemunaitis, J. (2022). NF- κ B signaling in tumor pathways focusing on breast and ovarian cancer. *Oncology Reviews*, 16, 10568. <https://doi.org/10.3389/or.2022.10568>

Falvo, J. V., Tsytsykova, A. V., & Goldfeld, A. E. (2010). Transcriptional control of the TNF gene. *Current Directions in Autoimmunity*, 11, 27–60. <https://doi.org/10.1159/000289196>

Garlanda, C., Dinarello, C. A., & Mantovani, A. (2013). The interleukin-1 family: Back to the future. *Immunity*, 39(6), 1003–1018. <https://doi.org/10.1016/j.immuni.2013.11.010>

Goldstein, D. A., Chen, Q., Ayer, T., Howard, D. H., Lipscomb, J., El-Rayes, B. F., & Flowers, C. R. (2015). First- and second-line bevacizumab in addition to chemotherapy for metastatic colorectal cancer: A United States-based cost-effectiveness analysis. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, 33(10), 1112–1118. <https://doi.org/10.1200/JCO.2014.58.4904>

Hamilton-Miller, J. M. (1973). Chemistry and biology of the polyene macrolide antibiotics. *Bacteriological Reviews*, 37(3), 166–196. <https://doi.org/10.1128/membr.37.3.166-196.1973>

Lacoviella, L., Bonaccio, M., de Gaetano, G., & Donati, M. B. (2020). Epidemiology of breast cancer, a paradigm of the “common soil” hypothesis. *Seminars in Cancer Biology*, 72, 4–10. <https://doi.org/10.1016/j.semcancer.2020.02.010>

Liu, K., Liu, P., Liu, R., & Wu, X. (2015). Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. *Medical Science Monitor Basic Research*, 21, 15–20. <https://doi.org/10.12659/MSMBR.893327>

Malvia, S., Bagadi, S. A., Dubey, U. S., & Saxena, S. (2017). Epidemiology of breast cancer in Indian women. *Asia-Pacific Journal of Clinical Oncology*, 13(4), 289–295. <https://doi.org/10.1111/ajco.12661>

Masuda, H., Zhang, D., Bartholomeusz, C., Doihara, H., Hortobagyi, G. N., & Ueno, N. T. (2012). Role of epidermal growth factor receptor in breast cancer. *Breast Cancer Research and Treatment*, 136(2), 331–345. <https://doi.org/10.1007/s10549-012-2289-9>

Matro, J. M., Li, T., Cristofanilli, M., Hughes, M. E., Ottesen, R. A., Weeks, J. C., & Wong, Y.-N. (2015). Inflammatory breast cancer management in the national comprehensive cancer network: The disease, recurrence pattern, and outcome. *Clinical Breast Cancer*, 15(1), 1–7. <https://doi.org/10.1016/j.clbc.2014.05.005>

Mercogliano, M. F., Bruni, S., Elizalde, P. V., & Schillaci, R. (2020). Tumor Necrosis Factor α Blockade: An Opportunity to Tackle Breast Cancer. *Frontiers in Oncology*, 10, 584. <https://doi.org/10.3389/fonc.2020.00584>

Nedeljković, M., & Damjanović, A. (2019). Mechanisms of chemotherapy resistance in triple-negative breast cancer—how we can rise to the challenge. *Cells*, 8(9). <https://doi.org/10.3390/cells8090957>

Obidiro, O., Battogtokh, G., & Akala, E. O. (2023a). Triple negative breast cancer treatment options and limitations: Future outlook. *Pharmaceutics*, 15(7), 1796. <https://doi.org/10.3390/pharmaceutics15071796>

Obidiro, O., Battogtokh, G., & Akala, E. O. (2023b). Triple negative breast cancer treatment options and limitations: Future outlook. *Pharmaceutics*, 15(7), 1796. <https://doi.org/10.3390/pharmaceutics15071796>

Orrantia-Borunda, E., Anchondo-Núñez, P., Acuña-Aguilar, L. E., Gómez-Valles, F. O., & Ramírez-Valdespino, C. A. (2022). Subtypes of breast cancer. In *Breast Cancer* (pp. 31–42). Exon Publications. <http://dx.doi.org/10.36255/exon-publications-breast-cancer-subtypes>

Ortega, M. A., Fraile-Martínez, O., Asúnsolo, Á., Buján, J., García-Honduvilla, N., & Coca, S. (2020). Signal transduction pathways in breast cancer: The important role of pi3k/akt/mtor. *Journal of Oncology*, 2020, 9258396. <https://doi.org/10.1155/2020/9258396>

Park, H. S., Jang, M. H., Kim, E. J., Kim, H. J., Lee, H. J., Kim, Y. J., Kim, J. H., Kang, E., Kim, S.-W., Kim, I. A., & Park, S. Y. (2014). High EGFR gene copy number predicts poor outcome in triple-negative breast cancer. *Modern Pathology*, 27(9), 1212–1222. <https://doi.org/10.1038/modpathol.2013.251>

Pavitra, E., Kancharla, J., Gupta, V. K., Prasad, K., Sung, J. Y., Kim, J., Tej, M. B., Choi, R., Lee, J.-H., Han, Y.-K., Raju, G. S. R., Bhaskar, L., & Huh, Y. S. (2023). The role of NF- κ B in breast cancer initiation, growth, metastasis, and resistance to chemotherapy. *Biomedicine & Pharmacotherapy*, 163, 114822. <https://doi.org/10.1016/j.biopha.2023.114822>

Pulido, C., Vendrell, I., Ferreira, A. R., Casimiro, S., Mansinho, A., Alho, I., & Costa, L. (2017). Bone metastasis risk factors in breast cancer. *Ecancermedicalscience*, 11, 715. <https://doi.org/10.3332/ecancer.2017.715>

Quest, A. F. G., Leyton, L., & Párraga, M. (2004). Caveolins, caveolae, and lipid rafts in cellular transport, signaling, and disease. *Biochemistry and Cell Biology*, 82(1), 129–144. <https://doi.org/10.1139/o03-071>

Rai, A., Misra, S. R., Panda, S., Sokolowski, G., Mishra, L., Das, R., & Lapinska, B. (2022). Nystatin effectiveness in oral candidiasis treatment: A systematic review & meta-analysis of clinical trials. *Life (Basel, Switzerland)*, 12(11), 1677. <https://doi.org/10.3390/life12111677>

Ranjitkar, S., Zhang, D., Sun, F., Salman, S., He, W., Venkitanarayanan, K., Tulman, E. R., & Tian, X. (2021). Cytotoxic effects on cancerous and non-cancerous cells of trans-cinnamaldehyde, carvacrol, and eugenol. *Scientific Reports*, 11(1). <https://doi.org/10.1038/s41598-021-95394-9>

Réb  , C., & Ghiringhelli, F. (2020). Interleukin-1   and cancer. *Cancers*, 12(7), 1791. <https://doi.org/10.3390/cancers12071791>

Richje, R. C., & Swanson, J. O. (2003). Breast cancer: a review of the literature. *J Insur Med*, 35(2), 85–101. <https://doi.org/PMID: 14733031>

Sathishkumar, K., Sankarapillai, J., Mathew, A., Nair, R. A., Gangane, N., Khuraijam, S., Barmon, D., Pandya, S., Majumdar, G., Deshmane, V., Zomawia, E., Bhutia, T. W., Jerang, K., George, P. S., Maliye, S., Laishram, R., Das, G., Shah, A., Debbarma, S., ... Mathur, P. (2024). Breast cancer survival in India across 11 geographic areas under the National Cancer Registry Programme. *Cancer*. <https://doi.org/10.1002/cncr.35188>

Scheller, J., & Rose-John, S. (2006). Interleukin-6 and its receptor: From bench to bedside. *Medical Microbiology and Immunology*, 195(4), 173–183. <https://doi.org/10.1007/s00430-006-0019-9>

Sherbiny, F. F., Bayoumi, A. H., El-Morsy, A. M., Sobhy, M., & Hagra, M. (2021). Design, Synthesis, biological Evaluation, and molecular docking studies of novel Pyrazolo[3,4-d]Pyrimidine derivative scaffolds as potent EGFR inhibitors and cell apoptosis inducers. *Bioorganic Chemistry*, 116, 105325. <https://doi.org/10.1016/j.bioorg.2021.105325>

Singh, K., Bhoir, M., Kasu, Y. A., Bhat, G., & Marar, T. (2018). Antioxidants as precision weapons in war against cancer chemotherapy induced toxicity - Exploring the armoury of obscurity. *Saudi Pharmaceutical Journal : SPJ : The Official Publication of the Saudi Pharmaceutical Society*, 26(2), 177–190. <https://doi.org/10.1016/j.jsps.2017.12.013>

(U.S.), N. I. of D. and D. and K. D. (2012). LiverTox: Clinical and Research Information on Drug-induced Liver Injury. National Institute of Diabetes and Digestive and Kidney Diseases.

Williams, M. M., & Cook, R. S. (2015). Bcl-2 family proteins in breast development and cancer: Could Mcl-1 targeting overcome therapeutic resistance? *Oncotarget*, 6(6), 3519–3530. <https://doi.org/10.18632/oncotarget.2792>

Williams, T. M., & Lisanti, M. P. (2004). The caveolin proteins. *Genome Biology*, 5(3), 1–8. <https://doi.org/10.1186/gb-2004-5-3-214>

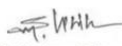
Cancer of the Breast (Female) - Cancer Stat Facts. (n.d.). SEER. Retrieved March 26, 2024, from <https://seer.cancer.gov/statfacts/html/breast.html>

Female Breast Cancer Subtypes - Cancer Stat Facts. (n.d.). SEER. Retrieved March 26, 2024, from <https://seer.cancer.gov/statfacts/html/breast-subtypes.html>

World Health Organization: WHO. (2024, March 13). Breast cancer. World Health Organization: WHO. <https://www.who.int/news-room/fact-sheets/detail/breast-cancer>

ANNEXURE

Presentation at the Monash Virtual Conference (December 2023) and the published abstract in the Abstract book

 CERTIFICATE OF PARTICIPATION This certifies that FATHIMA SUMAIRA S Uniting Minds in Global Health Sciences Monash Virtual Conference 2023 Jeffrey Cheah School of Medicine & Health Sciences 14 & 15 December 2023  Professor Dr. Shajahan Yasin Head of the School Jeffrey Cheah School of Medicine and Health Sciences Monash University Malaysia  Dr. Vetriselvan Subramaniam Organizing Chairman Jeffrey Cheah School of Medicine and Health Sciences Monash University Malaysia	(P012) Targeting the Apoptosis Regulator Bcl-2 Family Against a Derivative of the Milk Thistle Plant (<i>Silybum Marianum</i>) Fathima Sumaira, S Sathyabama Institute of Science and Technology Many factors develop cancer, one of the most lethal diseases on the planet. One such factor is B-cell lymphoma 2 (BCL-2) protein family members, also known as apoptosis regulators, which have a significant role in regulating apoptotic cell death and may develop resistance to cell death, a hallmark of cancer. So, BCL-2 protein family members can mediate through traditional plant compounds, unfurling the doorway to more convincing and rigorous targeted therapies. Silymarin, a derivative from the Milk Thistle plant (<i>Silybum marianum</i>), is an existing persuasive substance that possesses various pharmacological properties, which are currently being investigated as a lead compound for modulating apoptosis through targeting the BCL-2 family members. Using computational methods, we conduct molecular docking analyses to explore the interaction between Silymarin and BCL-2 family proteins, including pro- and anti-apoptotic members. The study integrates in silico prediction tools to assess molecular and pharmacokinetic properties and toxicological analyses offering insights into the compound. This analysis provides a nuanced understanding of the ability of the compound to modulate apoptosis through specific interactions with BCL-2 family members. In conclusion, this study contributes valuable insights into the molecular interactions, pharmacokinetics, and toxicological aspects of Silymarin, providing a foundation for further experimental validations and potential applications in apoptosis regulation. 20
---	---

GATE SCORE CARD

 GRADUATE APTITUDE TEST IN ENGINEERING 2024 अभियांत्रिकी स्नातक अभिक्षमता परीक्षा २०२४ ORGANISING INSTITUTE: INDIAN INSTITUTE OF SCIENCE, BENGALURU	
SCORE CARD	
Name of the Candidate FATHIMA SUMAIRA S	
Name of the Parent/Guardian SHAKIR M I	
Registration No.	BT24557402052
Test Paper	Biotechnology (BT)
Date of Examination	February 10, 2024
GATE Score	486
Marks out of 100	48.0
All India Rank (AIR) in the test paper	947
Qualifying Marks	
General	38.9
EWS/OBC-NCL	35.0
SC/ST/PwD	25.9
Number of candidates appeared for the test paper	17078
 Prof. Chandra Sekhar Seelamantula Organising Chairperson, GATE 2024 On behalf of NCB-GATE Ministry of Education (MoE)	
 cd7f76a49692902996d3c92b8ef151bd	
 	
A candidate is considered qualified if the marks secured are greater than or equal to the qualifying marks mentioned for the category, for which a valid category certificate, if applicable, must be produced along with this Score Card.	
This Score Card is valid up to 31 st March 2027.	

PLAGIARISM CHECK REPORT

RE-2022-225960 - Turnitin Plagiarism Report

by Fathima Sumaira

RE-2022-225960-plag-report

ORIGINALITY REPORT

20 %	16 %	13 %	4 %
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.ncbi.nlm.nih.gov Internet Source	2 %
2	www.mdpi.com Internet Source	1 %
3	www.frontiersin.org Internet Source	1 %
4	"Breast Cancer: From Bench to Personalized Medicine", Springer Science and Business Media LLC, 2022 Publication	1 %
5	www.sabcs.org Internet Source	1 %
6	link.springer.com Internet Source	1 %



Name of the student : Fathima Sumaira S

Register Number : 40230010

Title of the Thesis : Revitalising Nystatin for targeting cell proliferation and gene expression in breast cancer studies.

Internal Guide Name : Dr. Ramesh Kumar V

External Guide Name : Dr. Bavanilatha M

PO / PSO / PEO / SDG	Justification
PSO 2	Designing a system to repurpose nystatin against breast cancer involves strategic planning of drug repurposing protocols and molecular studies for efficacy validation.
PSO 3	Developing and conducting experiments to repurpose nystatin against breast cancer involves testing hypotheses regarding its efficacy, analyzing cell viability, and interpreting data to validate its potential as a treatment.
PO 4	Conducting investigations of complex technological problems in repurposing nystatin against breast cancer entails designing multifaceted experiments, analyzing diverse datasets, and interpreting findings to elucidate its therapeutic potential and optimize treatment strategies.
PO 5	Utilizing modern tools like molecular docking is crucial in repurposing nystatin against breast cancer. Docking helps predict the binding affinity of nystatin with cancer targets, aiding in rational drug design and optimization.