

TARGETING BREAST CANCER USING LINOLEIC ACID AND ITS EFFECT ON INFLAMMATORY GENE AND EXPRESSION STUDIES

PROJECT WORK

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

By

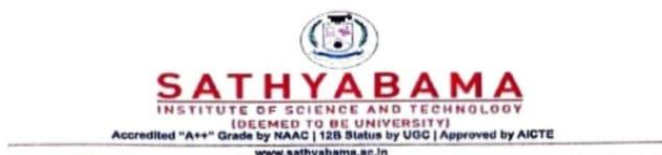
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BONAFIDE CERTIFICATE

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ABSTRACT

Breast cancer stands as one of the most prevalent causes of mortality among women worldwide, emphasizing the urgent need for innovative approaches to combat this formidable disease. In this context, ongoing research delves into the potential of linoleic acid, an omega-6 fatty acid found in various dietary sources, to impede the growth of breast cancer cells through its influence on inflammatory gene expression. Chronic inflammation emerges as a pivotal driver in the initiation and progression of breast cancer, underlining the significance of exploring interventions that target inflammatory pathways. Linoleic acid exhibits notable anti-inflammatory properties, exerting its effects through the modulation of key transcription factors such as NF- κ B and PPAR- γ . By regulating the activity of these transcription factors, linoleic acid holds the potential to alter the expression of genes intricately involved in inflammation, thereby disrupting the inflammatory milieu within the breast cancer microenvironment. Central to this investigation is the exploration of the complex interplay between linoleic acid and inflammatory gene expression in breast cancer cells. Specifically, the study scrutinizes the mechanisms through which inflammatory genes, including NF- κ B, TNF- α , IL-1 β , and IL-6, contribute to the progression of breast cancer. These genes play multifaceted roles in fostering tumor growth, angiogenesis, metastasis, and immune evasion, thereby exacerbating the malignancy of breast cancer. Furthermore, the research endeavors to elucidate the therapeutic potential of targeting these inflammatory pathways using linoleic acid as a dietary intervention. By dissecting the intricate molecular mechanisms underlying the crosstalk between linoleic acid and inflammatory gene expression, this study aims to identify novel therapeutic strategies for combating breast cancer.

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ABRREVIATION

PPAR γ	Peroxisome Proliferative Activated
NF κ B	Nuclear Factor kappa-B
TNF α	Tumour Necrosis Factor Alpha
IL-1 β	Interleukin-1 Beta
IL-6	Interleukin-6
PR	Progesterone Receptor
ER	Estrogen Receptor
IGF	Insulin like Growth Factor
HER 2	Human Epidermal Growth Factor Receptor 2
DCIS	Ductal Carcinoma In-Situ
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma
TNBC	Triple Negative Breast Cancer
NCRP	National Cancer Registry Programme
ICMR	Indian Council Medical Research

IKB	Inhibitor of kappa-B
IKK	IKB kinase
EGFR	Epidermal Growth Factor Receptor
BAFFR	B-cell Activating Factor Receptor
RANKL	Receptor Activator of Nuclear Factor kappa-B Ligand
NIK	NFkB Inducing Kinase
VEGFR	Vasco Epidermal Growth Factor Receptor
RNA	Ribo Nucleic Acid
cDNA	Complementary Deoxy-ribo Nucleic Acid
RT	Reverse Transcriptase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
IC 50	Half-maximal Inhibitory Concentration
AO	Acridine Orange
EtBr	Ethidium Bromide
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase

BBB

Blood Brain Barrier

PAINS

Pan Assay Interference Compounds

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CHAPTER 1

INTRODUCTION

Cancer is a complex and often devastating disease that affects millions of people worldwide. In simple terms, cancer refers to a group of more than 100 diseases that develop over time and involve the uncontrolled division of the body's cell ((US) & Study, 2007). Cancer cells develop because of multiple changes in their genes, which can have many possible causes, including lifestyle habits and inherited genes (What Is Cancer? | Cancer Basics | American Cancer Society, n.d.). These gene mutations turn normal cells into cancer cells, which can grow uncontrollably and spread to other parts of the body (Understanding Cancer - NCI, n.d.). While cancer can start in almost any organ or tissue of the body, it is typically classified into two broad categories: hematologic (blood) cancers and solid tumour cancers (What Is Cancer? | Cancer Basics | American Cancer Society, n.d.).

Some of the most common types of cancer include non-melanoma skin cancer, lung cancer, prostate cancer, breast cancer, and colorectal cancer. Each type of cancer has its unique set of symptoms, which may include changes in bowel or bladder habits, unexplained weight loss, persistent cough or hoarseness, and unusual bleeding or discharge. Early detection is crucial to successful treatment, and cancer screening and early detection aim to identify the malignancy or its precursor lesion at an early stage (Loud & Murphy, 2017). Screening in both healthy and high-risk populations offer the opportunity to detect cancer early, leading to an increased opportunity for treatment and survival (Schiffman *et al.*, 2015). The treatment options for cancer depend on the type and stage of cancer, as well as the individual's overall health and personal preferences. Common treatments include surgery, radiation therapy, chemotherapy, immunotherapy, and targeted therapy. While cancer can be a frightening and overwhelming diagnosis, it's important to remember that many people survive and even thrive after cancer treatment (Crosby *et al.*, 2022). With advances in research and treatment, there is hope for those affected by cancer, and understanding the disease and its treatment options is an important step in fighting back against cancer. The World Health Organization

(WHO) estimates that there will be 10 million cancer-related deaths and 19.3 million new cases of the disease globally in 2020, making cancer a significant health burden. These statistics are expected to rise significantly over the next several decades as a result of aging populations, shifting lifestyles, and environmental changes. All ages, races, and socioeconomic backgrounds are affected by cancer, but low- and middle-income nations are more affected than others because they may have less access to early detection, diagnosis, and treatment services.

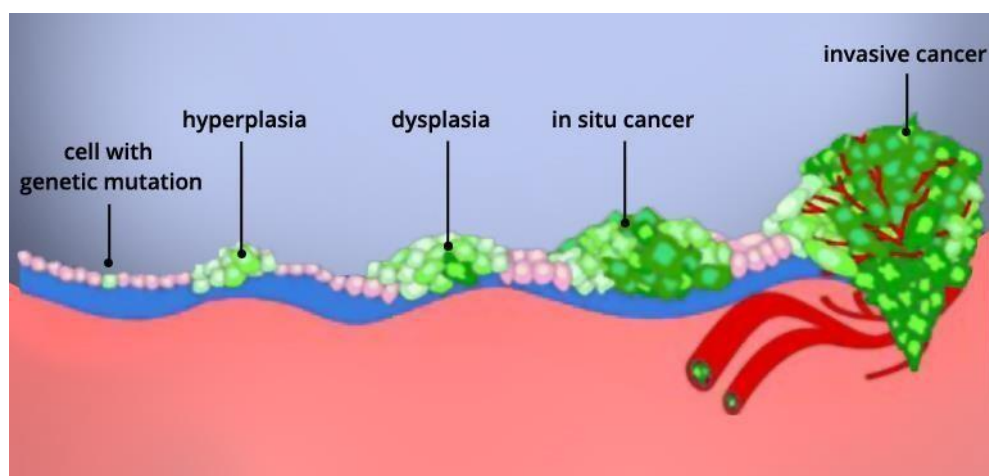


Figure 1.1: Tumor Development

Breast cancer is an alarming and lethal disease in the world (Kaltschmidt *et al.*, 2022). The biggest cause of cancer-related deaths in women globally is breast cancer. They affect women who have a family history of breast cancer and make up about 2.5% of all breast cancer cases. Although breast cancer can affect both men and women, it affects women more frequently than it does, with women making up the majority of cases. GLOBOCAN estimates that this cancer causes 522,000 deaths in women annually. The danger of metastasizing, or the spread of cancer cells from the main tumor to nearby tissues and distant organs, increases with a late diagnosis. 90% of mortality from solid tumors are caused by metastasis, which is the cause of practically all deaths from breast cancer (Pires *et al.*, 2017). Annually, 225,000 new cases of invasive breast cancer are discovered in the United States, while ductal carcinoma in situ (DCIS) is detected in at least 50,000 women (Wu & Kral,

2005). The development of breast cancer is influenced by both hereditary and nongenetic factors (Biswas & Iglehart, 2006).

Aging, reproductive variables (such as menarche, menopause, and childbearing age), obesity, alcohol consumption, physical exercise and bacterial infection are examples of non-genetic factors (Takeuchi & Gotoh, 2023). Breast cancer accounts for about 27% of all female cancer cases, making it the most frequent malignancy among Indian women, according to the Indian Council of Medical Research (ICMR). The increased prevalence of breast cancer in India can be attributed to various reasons, such as evolving lifestyle patterns, urbanization, postponing childbirth, and embracing Western eating practices. Timely diagnosis and treatment are further complicated by cultural taboos around women's health, low knowledge of breast cancer, and restricted access to screening and early detection services. In India, there are notable geographical differences in the incidence of breast cancer, according to data from the National Cancer Registry Programme (NCRP). With over 27% of all cancer cases in women diagnosed annually, breast cancer is the most frequent cancer among women in India. With rising incidence rates seen in recent years, breast cancer is becoming a more significant public health concern in India. Although the prevalence of breast cancer in India is still lower than in Western nations, Indian women are disproportionately affected by the disease at a younger age, frequently during their reproductive years. States like Maharashtra, Kerala, and Delhi have been found to have higher rates of breast cancer incidence. Many Indian women still encounter obstacles to receiving high-quality healthcare, which delays diagnosis and results in less favourable outcomes. This is true even in the face of attempts to strengthen the infrastructure for cancer care and raise awareness of the disease.

Breast cancer can also be classified into different subtypes based on various factors, including hormone receptor status (Estrogen receptor [ER], Progesterone Receptor [PR]) and Human Epidermal Growth Factor Receptor 2 (HER2) expression (Slamon *et al.*, 2001). Another Breast cancer subtypes, each with distinct characteristics include:

1. Ductal Carcinoma In-Situ (DCIS): This subtype of breast cancer is characterized by abnormal cells that are confined to the milk ducts and have not invaded surrounding tissues. While DCIS is non-invasive, it may progress to invasive breast cancer if left untreated.
2. Invasive Ductal Carcinoma (IDC): IDC is the most common type of breast cancer, accounting for approximately 80% of all cases. It occurs when cancer cells invade surrounding breast tissue beyond the ducts, potentially spreading to nearby lymph nodes and other organs.
3. Invasive Lobular Carcinoma (ILC): This subtype originates in the lobules of the breast and accounts for approximately 10-15% of all breast cancer cases. ILC tends to be less common and more challenging to detect than IDC, as it may not form a distinct lump and can spread diffusely throughout the breast tissue.
4. Triple-Negative Breast Cancer (TNBC): TNBC is a subtype of breast cancer characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression. It tends to be more aggressive and has fewer targeted treatment options compared to other subtypes.

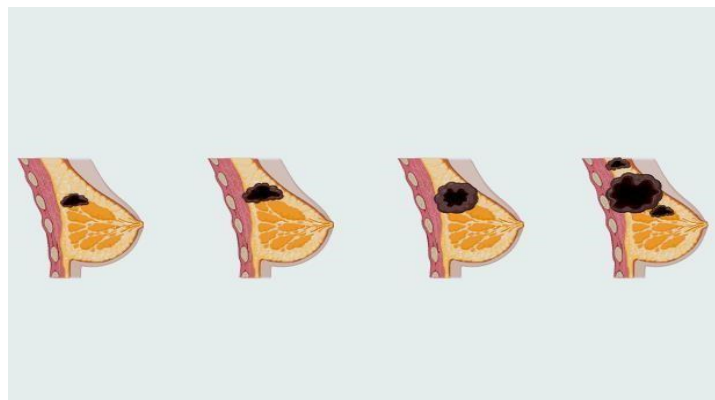


Figure 1.2: Breast Cancer Development

The purpose of this research, "Targeting Breast Cancer Using Linoleic Acid and Its Effect on Inflammatory Gene Expression and Expression Studies," is to shed light on whether linoleic acid inhibits the growth of cancer cells, namely by affecting the expression of genes that are associated with inflammation. One important nutrient that is plentiful in many dietary sources,

including nuts, seeds, and vegetable oils, is linoleic acid, a polyunsaturated omega-6 fatty acid. In along with controlling inflammatory responses and modifying gene expression, it is essential for preserving the integrity of cellular membranes. With regard to cancer prevention and therapy, linoleic acid has shown promise recently, especially when it comes to breast cancer.

An increasing amount of experimental and clinical data has led to the focus on linoleic acid's possible anticancer effects. By regulating the expression of inflammatory genes and cytokines linked to the development of cancer, linoleic acid has antiinflammatory properties. The development and metastasis of breast cancer are significantly influenced by chronic inflammation, and linoleic acid's capacity to block inflammatory signaling pathways may prevent tumor growth and invasion. Apoptosis and cell division, two essential processes that are dysregulated in cancer, are influenced by linoleic acid. Research has indicated that linoleic acid has the ability to cause cell cycle arrest and death in breast cancer cells, thereby impeding their ability to proliferate and endure. Furthermore, linoleic acid may make cancer cells more sensitive to traditional treatments, which would increase their effectiveness in the fight against breast cancer. A considerable fraction of breast cancer cases is estrogen receptor (ER)-positive, and endocrine therapy is frequently used as a treatment. It is possible that linoleic acid can prevent ER-positive breast cancer by modifying the metabolism of estrogen and signaling pathways. Additionally, the anticancer effects of linoleic acid may be attributed to its interactions with other hormonal pathways, including insulin-like growth factor (IGF) signaling. The development of new blood vessels, or angiogenesis, is necessary for the growth and metastasis of tumors. It has been demonstrated that linoleic acid inhibits angiogenic activities and decreases the production of new blood vessels in breast tumors, thereby impairing their ability to grow and spread to distant sites. In addition, by focusing on the invasive and migratory abilities of cancer cells, linoleic acid may disrupt the mechanisms involved in metastasis. When used with other anticancer medications, linoleic acid may have synergistic effects that increase their effectiveness against breast cancer. Preclinical studies have

demonstrated encouraging outcomes for combination techniques utilizing linoleic acid and conventional chemotherapeutic medicines or targeted therapies, underscoring the promise of these approaches in clinical settings (den Hartigh, 2019).

Predicting the atomic-level binding interactions between a tiny molecule (such a drug candidate) and its target protein is possible through the use of strong computational methods called *in silico* docking studies in drug discovery and development. For the purpose of locating possible therapeutic candidates, clarifying their modes of action, and maximizing their binding affinity and specificity, this methodology is especially beneficial. For the purpose of developing new treatments that target certain biochemical pathways involved in the growth and spread of cancer, *in silico* docking studies are essential in the field of cancer research.

Using molecular docking simulations and other *in silico* studies to target nuclear factor kappa B (NF- κ B), a crucial transcription factor implicated in inflammatory signaling pathways, is one of the main components of this investigation. Peroxisome proliferator-activated receptor gamma (PPAR- γ) is a strategically chosen ligand for docking investigations since linoleic acid is an extracellular agent. PPAR- γ is a nuclear receptor that has been linked to the control of NF- κ B signaling and is known to modify the expression of genes related to inflammation. It is postulated that linoleic acid targeting PPAR- γ may have downstream effects on the activity of the NF- κ B pathway, affecting the expression of genes involved in inflammation and contributing to the advancement of breast cancer. The intricate interaction of several variables in breast cancer genesis, progression, and therapy resistance continues to provide considerable challenges despite major breakthroughs in treatment approaches. An increasing body of research in recent years points to the critical role that inflammatory processes play in the development and spread of breast cancer. The expression of inflammatory genes, which include important actors like NF- κ B, TNF- α , IL-1 β , and IL-6, has been intimately associated with breast cancer metastasis, carcinogenesis, and resistance to treatment. As a result, focusing on inflammatory pathways has become a viable treatment approach.

Furthermore, a great deal of research has been paid to the effects of food components on the risk and progression of breast cancer (Danforth, 2021).

Linoleic acid is an omega-6 fatty acid that is widely available in food sources has been linked to the control of inflammatory responses. There is substantial therapeutic potential in comprehending the intricate connection that exists between linoleic acid and inflammatory gene expression in breast cancer cells. In this thorough analysis, we explain the impact of linoleic acid on inflammatory pathways, examine the complex mechanisms behind the association between inflammatory gene expression and breast cancer, and consider the potential therapeutic benefits of focusing on these pathways for the treatment of breast cancer.

Nuclear Factor kappa-B cells, or NF- κ B cells, are at the forefront of inflammatory signaling pathways and have a complex function in the development of breast cancer. NF κ B constitutive activation has been linked to aggressive tumor behaviour, metastasis, and treatment resistance in a number of breast cancer subtypes. Tumor growth and progression are significantly impacted by NF- κ B, which controls the expression of genes related to angiogenesis, apoptosis evasion, inflammation, and cell proliferation. The pro-inflammatory cytokine tumor necrosis factor-alpha (TNF- α), which is predominantly produced by immune cells, has been linked to the development of breast cancer. Angiogenesis, metastasis, and tumor aggressiveness are all correlated with elevated levels of TNF- α in the tumor microenvironment. By activating downstream signaling pathways such NF κ B, MAPK, and PI3K/Akt, TNF- α facilitates the survival, proliferation, and migration of cancer cells. Interleukin-1 (IL-1), which consists of isoforms IL-1 α and IL-1 β , has a variety of pleiotropic effects on immune response, inflammation, and carcinogenesis (Esquivel-Velázquez *et al.*, 2015).

By accelerating angiogenesis, encouraging the epithelial-to-mesenchymal transition (EMT), and boosting cancer cell invasiveness, IL-1 β , signaling aids in the tumor's growth in breast cancer. Furthermore, IL-1 β ,

promotes the activation of immune cells within the tumor microenvironment, creating an inflammatory environment that supports the development and spread of tumors. The initiation and progression of breast cancer are significantly influenced by interleukin-6 (IL-6), a critical mediator of inflammation and immunological control. In individuals with breast cancer, elevated levels of IL-6 are linked to worse tumor invasiveness, a worse prognosis, and treatment resistance. Cancer cell survival, growth, and metastasis are all aided by IL-6.

The diet is rich in omega-6 polyunsaturated fatty acids, such as linoleic acid (LA), which has been linked to regulating inflammatory responses by interacting with NF- κ B signaling. Research findings indicate that NF- κ B activity can be either activated or inhibited by linoleic acid, contingent on cellular context, concentration, and exposure length. Tumor growth and metastasis have been linked to increased expression of proinflammatory cytokines, chemokines, and adhesion molecules in breast cancer cells, which is caused by linoleic acid-mediated activation of NF- κ B. On the other hand, linoleic acid has been shown in some studies to have anti-inflammatory properties, whereby it inhibits NF- κ B activation and reduces the expression of inflammatory mediators in breast cancer cells. TNF- α signaling and linoleic acid have a complex, reciprocal relationship. TNF- α has the ability to increase inflammatory responses and accelerate the growth of tumors by stimulating the release of linoleic acid from adipocytes and facilitating its absorption into cellular membranes. Conversely, it has been demonstrated that linoleic acid controls TNF- α expression by modifying transcriptional and post-transcriptional pathways. Research indicates that elevated levels of linoleic acid could trigger TNF- α production by means of NF- κ B activation. The expression patterns and functional implications of inflammatory gene dysregulation in breast cancer have been studied using cell culture systems. These models serve as a platform for assessing the effectiveness of targeted therapies, such as linoleic acid supplementation, and they provide insightful information about the molecular pathways underlying tumor-associated inflammation (Brierly *et al.*, 2023).

The complex relationship that exists between the expression of inflammatory genes and the development of breast cancer highlights the significance of determining the underlying mechanisms and investigating new approaches to treatment. Given its two functions in regulating inflammatory reactions, linoleic acid is a dietary component that could potentially use as a target for breast cancer treatment

CHAPTER 2

LITERATURE SURVEY

2.1 Introduction to Nuclear Factor Kappa B

The Nuclear Factor Kappa B (NF- κ B) activation pathway is a complex signaling pathway that regulates the expression of genes involved in immune responses, inflammation, cell proliferation, and survival. There are two pathways for NF- κ B activation: the canonical and non-canonical pathways. The canonical pathway is activated by a variety of stimuli, including proinflammatory cytokines, bacterial and viral products, and stress signals. It involves the phosphorylation and degradation of the inhibitor of kappa B (I κ B) proteins, which normally sequester NF- κ B in the cytoplasm. Upon I κ B degradation, NF- κ B translocates to the nucleus and binds to specific DNA sequences, resulting in the transcriptional activation of target genes (Zinatizadeh *et al.*, 2020). The non-canonical pathway, on the other hand, is activated by a distinct set of receptors, including the lymphotoxin beta receptor (LT β R), the B-cell activating factor receptor (BAFFR), and the receptor activator of nuclear factor kappa-B ligand (RANKL). It involves the processing of p100 to p52 and the formation of p52/RelB heterodimers, which regulate the expression of genes involved in lymphoid tissue development and immune responses to pathogens and tumors (Karin & Ben-Neriah, 2000).

Dysregulation of the NF- κ B pathway has been implicated in various diseases, including cancer, autoimmune disorders, and inflammatory diseases (Courtois *et al.*, 2016).

This two signaling pathways lead to the activation of NF- κ B known as the canonical pathway (classical) and the non-canonical pathway (alternative pathway). The common regulatory step in both of these cascades is activation of an I κ B kinase (IKK) complex consisting of catalytic kinase subunits (IKK α /IKK β) and the regulatory non-enzymatic scaffold protein NEMO (NF-kappa, B essential modulator also known as IKK γ). Activation of dimmers NF- κ B is due to IKK-mediated phosphorylation induced proteasomal degradation

of the I κ B inhibitor enabling the active NF- κ B transcription leads to the expression of the I κ B α gene, which consequently sequesters NF- κ B subunits and terminated transcriptional activity unless a persistent activation signal present. Misregulated NF- κ B activity is frequently seen in a variety of illnesses, such as cancer and chronic inflammation. Therefore, conserving human health demands an understanding of the manner in which NF- κ B signaling is modulated (Shih *et al.*, 2011). A family of inhibitors, which includes I κ B α and other related ankyrin repeat-containing proteins, generally sequesters the NF- κ B proteins in the cytoplasm. When NF- κ B 1 and NF- κ B2 are translated, they result in the precursor proteins p105 and p100, which act as NF- κ B inhibitors due to their I κ B-like C-terminal regions. When p105 and p100 are processed by the proteasome, the I κ B-like function of these precursor proteins is disrupted in addition to producing the mature NF- κ B1 and NF- κ B2 proteins (p50 and p52) (Basak *et al.*, 2007). The RHD functions as a mediator for dimerization, DNA binding, interaction with I κ Bs, and nuclear translocation within the five monomers RelA, RelB, cRel, p50, and p52. The five monomers combine to generate potential dimers. All monomer creation is transcriptionally controlled, although processing of the protein precursors p105 and p100, respectively, also controls the production of p50 and p52. As a result, unique NF- κ B dimeric complexes express differently depending on the cell type and stimulus. RelA:p50, cRel:p50, and RelB:p52 are a few of the physiologically noteworthy dimers. The single polypeptide I κ Bs: I κ B α , I κ B β and I κ B γ make up the traditional inhibitor proteins in the NF- κ B signaling cascade of events (Savinova *et al.*, 2009).

2.2 Canonical pathway

The canonical pathway (figure:1) typically represents that triggers the quick and reversible immunological and inflammatory manifestations, while the noncanonical pathway is usually what triggers the slower and irreversible developmental reactions (Shih *et al.*, 2009). The conventional pathway is outlined as being mediated by an IKK that is dependent on NEMO (IKK), whilst

the non-canonical pathway has been described as being mediated by an IKK1 and NF- κ B-inducing kinase (NIK) complex which is NEMO-independent (Beg *et al.*, 1995). Proinflammatory signals including cytokines, pathogen-associated molecular patterns (PAMPs), and some danger associated molecular patterns (DAMPs) all serve to activate the canonical NF κ B pathway. Extracellular and intracellular receptors activate NEMO-containing kinase complexes upon exposure of cells to these compounds by binding different proteins involved in the production of ubiquitin chains. Phosphorylation of serine (Ser) 177 and Ser181 causes IKK2 to switch from inactive to active. The traditional I κ Bs (I κ B α , I κ B β and I κ B ϵ) are phosphorylated and designated for degradation once canonical IKK is activated, releasing NF- κ B into the nucleus. The E3 ubiquitin ligase SCF/TRCP gets drawn to the phosphorylated N-terminal serines (Ser32/36) of the canonical I- κ B, designating it for degradation by the 26S proteasome. I κ B is ubiquitinated and deteriorated, which allows NF- κ B to get into the nucleus and trigger transcriptional activation. When the canonical IKK becomes active, similar phosphorylation on I κ B and IB occur, causing the degradation of these I κ Bs and the release of NF- κ B into the nucleus. It's interesting to note that the molecular processes underlying the canonical

IKK complex's enzymatic the precision for classical I- κ B proteins remained unknown. The principal NF- κ B effectors of the canonical route are polypeptides with transactivation domains. RelA/p65 and cRel are proteins that either form homodimers or heterodimers with p50, which lacks a transactivation domain. In response to stimuli of the canonical route, four transcriptional activators (RelA: RelA, RelA:p50, cRel:cRel, and cRel:p50) may be activated.

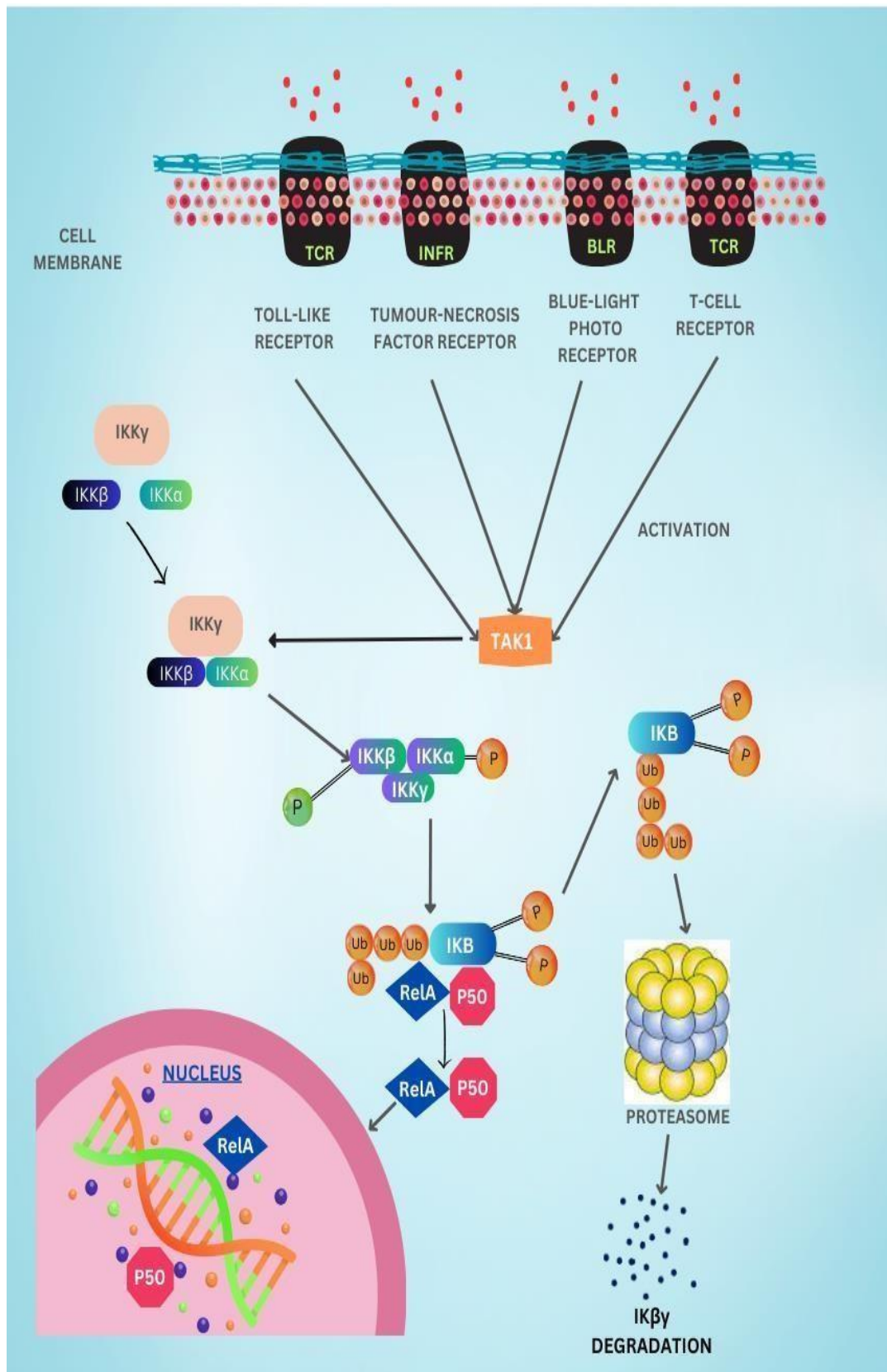


Figure 2.1: Canonical pathway of NF- κ B

2.3 Non-canonical pathway

The non-canonical pathway (figure:2) is one of the two pathways for NF- κ B activation, and it is activated by a distinct set of receptors, including the lymphotoxin beta receptor (LT β R), the B-cell activating factor receptor (BAFFR), and the receptor activator of nuclear factor kappa-B ligand (RANKL). Unlike the canonical pathway, which involves the degradation of I κ B α and the release of p65/p50 heterodimers, the non-canonical pathway involves the processing of p100 to p52 and the formation of p52/RelB heterodimers. The non-canonical pathway is important for the development and organization of lymphoid tissues, as well as for the regulation of immune responses to pathogens and tumors. Dysregulation of the non-canonical pathway has been implicated in various diseases, including autoimmune disorders, lymphomas, and osteoporosis (Brown *et al.*, 2008). The non-canonical pathway (figure:2) of inflammasome activation is mainly dependent on caspase-11, and also by caspase-4, caspase-5, and caspase-8. This pathway was recently discovered and is speculated to be homologous with murine caspase-11. Human caspase-4/caspase-5 can regulate the release of inflammatory cytokines by binding LPS directly via CARD domains in an LPS stimulated monocytes model (Xiang *et al.*, 2020). The non-canonical pathway is distinct from the canonical pathway, which is mainly dependent on caspase-1 (Lamkanfi & Dixit, 2012). The RelB/p52 NF- κ B complex is activated through a noncanonical NF- κ B pathway employing a mechanism that relies on the inducible processing of p100 rather than the degradation of I κ B α . Genetic data indicates that this NF- κ B pathway controls vital biological processes such lymphoid organogenesis, B-cell survival and maturation, dendritic cell activation, and bone metabolism. Therefore, a deeper comprehension of the mechanism governing non-canonical NF- κ B activation has significant therapeutic benefits. It grows more and more obvious that the signaling mechanisms used by this NF- κ B activation route are significantly different from those used by the canonical NF κ B pathway (Zinatizadeh *et al.*, 2020).

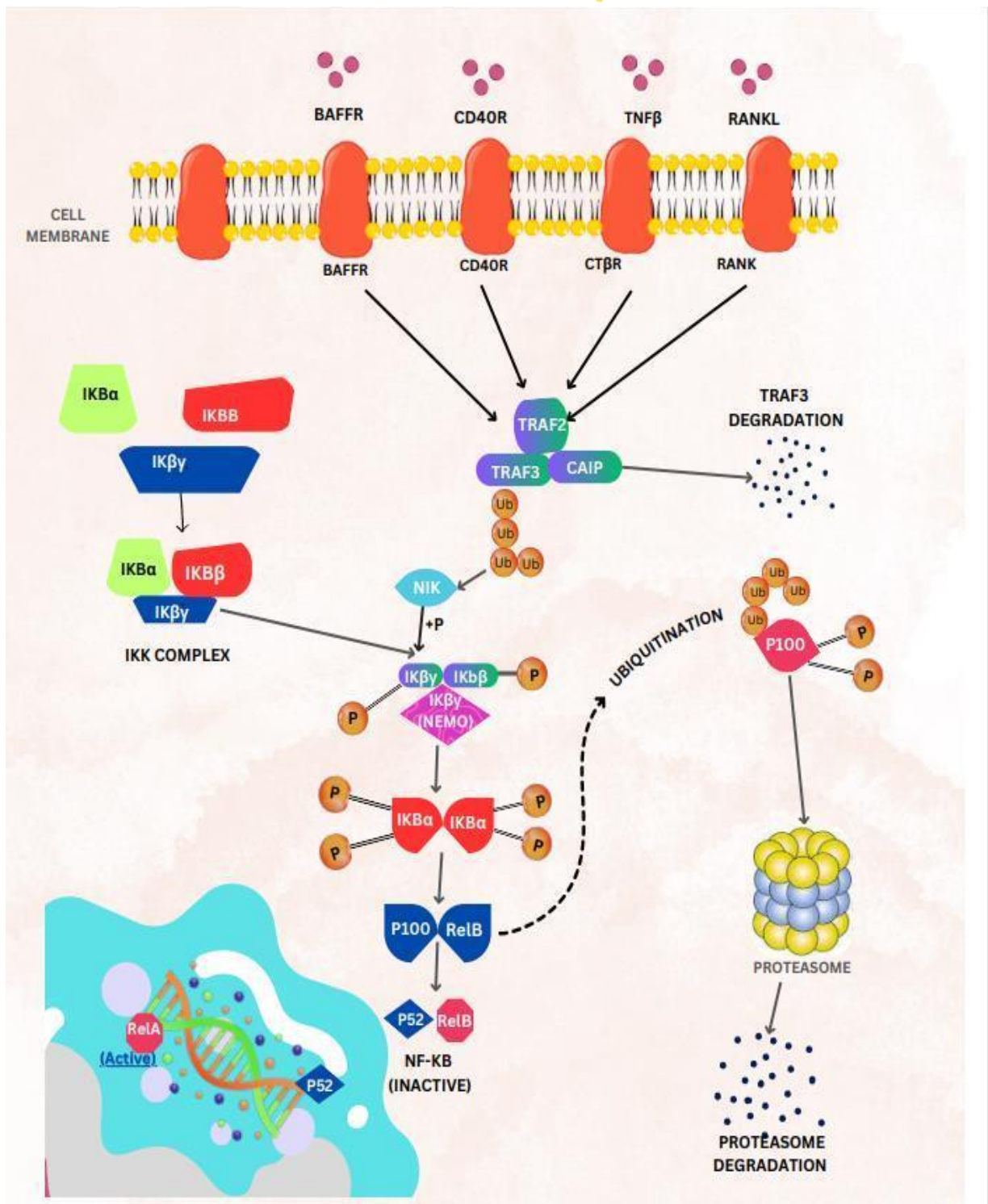


Figure 2.2: Non-canonical pathway of NF-κB

2.4 Nuclear factor kappa b in breast cancer

NF- κ B is “anti-apoptotic” and capable of enhancing cell survival in the face of stress (Barkett & Gilmore, 1999). NF- κ B is “pro-proliferative” due to its direct impact on genes essential for cell-cycle progression (such as cyclin D1) as well as by producing interleukins, cytokines, chemokines, and their corresponding receptors (Biswas *et al.*, 2001). NF- κ B was once thought to be only involved in immune system signaling, but it has recently come to our attention that it also acts as an upstream regulator of numerous other significant signaling pathways, including those involved in tumor promotion (Garg *et al.*, 2003). NF- κ B is also a crucial modulator of the apoptotic response of cells to damage, as has been shown by gene knockout studies. The development of breast cancer and how it responds to chemotherapy and radiation may both be impacted by NF- κ B regulation of the apoptotic pathway in cellular resistance to cytotoxic therapies. There is also mounting proof that NF- κ B contributes to the emergence of chemoresistance (Montagut *et al.*, 2006). NF- κ B signaling activation creates a feed-forward loop that promotes tumor growth and the inflammatory state in immune and cancer cells alike (Sica & Mantovani, 2012). The development of breast cancer may in part be caused by unchecked or abnormal NF- κ B activation. NF- κ B is an antagonist that prevents epidermal growth factor from activating NF- κ B and leads to the death of ER-breast cancer cells through apoptosis. Additionally, they demonstrated that the antagonist significantly reduced the size of ER-mouse mammary epithelial tumors and prevented their proliferation (Biswas *et al.*, 2001). Cancer genomics has proved to be useful in demonstrating the key role of NF- κ B in carcinogenesis and identifying vital steps that drive its activation (Nicolau *et al.*, 2007). Effective transcription of the target genes of NF- κ B activation depends on NF- κ B activity. More than 200 target genes have been demonstrated to be transcribed either directly or indirectly by NF- κ B. Many of these genes have crucial roles in the initiation, development, and metastasis of tumors. For instance, research conducted in vitro has suggested that aberrant NF- κ B control of the expression of cyclin D1 and c-myc may be involved in the early stages of breast cancer. NF- κ B might not be adequate for complete transcription, though, as additional transcription factors are also needed. While

a specific stimulus may activate NF- κ B, the target gene will not be fully transcribed if it does not also activate another transcription factor. Despite the enormous number of NF- κ B target genes, this combinatorial regulation of transcription partially enables the selective activation of particular genes under particular situations (Pahl, 1999).

Nuclear factor kappa b is first inactive in cytoplasm of the cell. It is activated by some inhibitors in two activation pathways (Khongthong *et al.*, 2019). There are two main mechanisms that control the activity of these proteins: the conventional or classical activation pathway and the alternative or non-canonical pathway. These proteins can form homodimer and heterodimeric complexes. Nuclear p65/p50 are the main components of the canonical route, which is triggered after IKK/IB phosphorylates IB and then degrades it. Specifically in canonical NF- κ B signaling, RELA/p50 or cREL/p50 heterodimers move to the nucleus in response to IL-1, TNF, or bacterial lipopolysaccharide (LPS), which activate TAK1/IKK and cause the phosphorylation and degradation of I κ B α . When Lymphotoxin Beta, Cluster of Differentiation 40 (CD40) ligand, or B-cell activating factor (BAFF) is present, the NF-B-inducing Kinase (NIK)/IKK complex phosphorylates RELB/p52 and translocates it into the nucleus (Oeckinghaus & Ghosh, 2009). In non-canonical pathway IKK homodimers that phosphorylate p100 to signal its partial proteolytic processing to mature p52, which translocates to the nucleus with RelB to mediate transcriptional regulation, control the alternative NF- κ B pathway (p100/p52). Proteolytic processing releases p50 and p52 from NF- κ B1 and NF- κ B2, respectively. Before activation, the NF- κ B subunits can either form homo- or heterodimers in the cytoplasm (Baldwin, 1996). IKB (inhibitor of KB) family members associate with NF- κ B dimers to sequester them in the cytoplasm in an inactive state (Yamazaki *et al.*, 2001). The inhibitor of NF- κ B (I κ B), which itself has five isomeric forms, including I κ B α , I κ B β , B κ B γ , I κ B ϵ , and BCL3, binds to NF- κ B dimers to prevent them from moving around the cytoplasm. I κ B is the most prevalent inhibitor protein for NF- κ B among the structurally related proteins known as I κ B-a, I κ B-b, I κ B-e, I κ B- α , I κ B β , p105, p100, and bcl3 that make up the I κ B family (Perou *et al.*, 2000).

Transactivation of the transcription factors Nuclear Factor Kappa B (NF- κ B) and Activator protein-1 (AP-1) is a key component of IL-1 signaling (mediating inflammation in response to a wide range of stimuli) (Franzoso *et al.*, 1992). In particular, the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) activate NF κ B in the immune cells. TNF, IL-1, IL-6, and vascular endothelial growth factor (VEGF) are examples of inflammatory cytokines and chemokines that are produced more readily when NF- κ B signaling is activated in immune cells (Diep *et al.*, 2022). By encouraging angiogenesis, epithelial to mesenchymal transition (EMT), the spread of circulating tumor cells (CTCs), invasion, and metastasis, IL-1 and NF- κ B signaling increase BCa tumor growth and progression. In the primary and metastatic environments, IL-1 is produced by a variety of different cell types, including cancer cells, macrophages, and osteoblasts to encourage tumorigenic behaviors such as chemotaxis, angiogenesis, and metastatic niche colonization (Zhao *et al.*, 2021).

Nuclear factor- κ B activation has both pro- and anti-tumorigenic effects in macrophages and T cells, indicating that the NF- κ B subunit's action is contextdependent. The polarization of the two types of tumor-associated macrophages (M1 and M2) is capable of being reversed (Murray, 2017). It has been noted that NF κ B p55 subunit activity controls macrophage polarization toward M1. Through NF- κ B signaling, M1 macrophages release inflammatory cytokines such TNF, IL-1, IL-2, IL-6, IL-12, and IL-23 and work to prevent tumor growth. A pro-tumorigenic role is played by the antiinflammatory mediators that M2 macrophages release, such as transforming growth factor (TGF) and IL-10. Effector T cells are stimulated by NF- κ B activation in T cells, and the quantity of IFN γ -producing CD8⁺ T cells that play a tumor-suppressive role is increased (Evaristo *et al.*, 2016). It is widely acknowledged that the presence of IL-1 or IL-1 correlates with aggressive disease characteristics such advanced stage, basal subtype, or metastasis in primary tumors or serum of Breast cancer patients (Voronov *et al.*, 2003).

The most frequent form of NF- κ B is a p65/RelA-p50 heterodimer, which is kept in an inactive state by its connection with the inhibitory I κ B α . AKT (involved in many cell cycles that controls cell proliferation, cell survival and motility) regulates transcriptional activity of nuclear κ B by degrading inhibitor of κ B (IKB) (Bai *et al.*, 2009). AKT, which generates a Pro survival protein that can be overexpressed in human breast cancer and can reduce the expression of the tumor suppressor p53 gene and the proapoptotic gene growth arrest and DNA damage (GADD)153, is another gene whose expression is controlled by NF- κ B (Benezra *et al.*, 2003).

Chemotherapeutic drugs, radiation, inflammatory cytokines, bacterial products, viral products, and oxidants are just a few of the stimuli that might cause I κ B α to be phosphorylated (Bharti & Aggarwal, 2002). The p65 (RelA) transcription factor is the most well-known member of the nuclear factor (NF)- κ B family, which is made up of closely related transcription factors (Bear *et al.*, 2003). NF- κ B controls P53, a crucial regulator of apoptosis and the cell cycle, both directly and indirectly. The direct impact of NF- κ B on the transcription of the gene mutated overrides the indirect regulation of p53 (Piret *et al.*, 1999). There is evidence that NF- κ B suppresses p53 activity. Cells with damaged DNA are known to undergo apoptosis when p53 is present, but NF- κ B encourages the survival of these altered cells, which results in the development of cancer. Similarly, it is shown that NF- κ B suppressed the GADD153/CHOP gene's expression, causing cells to undergo apoptosis when under stress. NF- κ B may increase the survival and transformation of cells with damaged DNA by decreasing the expression of this gene. A recent examination in human breast cancer cell lines and animal models of breast cancer about the activation and levels of NF- κ B associate compounds and from the result it is concluded that p100 subunit is overexpressed in the majority of breast cancer samples and breast cancer cell lines, suggesting a role for the precursor in sequestering others NF- κ B related proteins in cytoplasm. The majority of human breast cancer cell lines have activated RelA/p65 (RelA is also another form of nuclear factor κ B), which is linked to the transformation of breast cancer cells to hormone-independent proliferation, a trait of more aggressive and metastatic

tumors, breast tumor formation in animal models was linked to the overexpression of mouse cRel (Romieu-Mourez *et al.*, 2003).

When activated, the nuclear factor kappaB (NF- κ B) family of multifunctional transcription factors cause pleiotropic alterations in the target cells. Many human disorders, including breast tumors, have elevated levels of active NF- κ B. Specific subtypes of breast cancer as discussed above are shown to have high levels of active NF- κ B, primarily in ER-negative and EGFR, VEGFR overexpressing breast tumors (predominantly HER2 amplified tumors) (Pahl, 1999). IL-1 promotes tumor angiogenesis by upregulating Vascular Endothelial Growth Factor (VEGF) signaling in tumor cells and endothelial cells and by regulating endothelial cell function. Murine 4T1 BCa cells treated with leptin in the presence of mouse anti-IL1R antibody showed reduced VEGF and VEGF Receptor (VEGFR) mRNA and protein levels. C-X-C chemokine receptor type 7 (CXCR-7) induces endothelial progenitor cell (EPC) VEGF expression and tube formation; treatment with the IKK α /IKK β inhibitor, BAY 11-7082, blocked IL-1 β -NF- κ B-dependent CXCR-7 upregulation in EPCs. IL-1Ra or anakinra reduced tumor angiogenesis *in vivo*, where IL-1Ra inhibited proliferation of the HUVEC endothelial cell line *in-vitro* and inhibited MCF7 BCa xenograft tumor growth and vascularization *in-vivo*. Finally, IL-1 α and IL-1 β knockout mice showed reduced vascularization and growth of murine DA3 BCa tumors. Taken together, IL-1-NF- κ B is sufficient to induce angiogenesis by increasing VEGF (Vascular Endothelial Growth Factor) signaling in tumor cells and endothelial cells and controlling endothelial cell function, IL-1 encourages tumor angiogenesis. Leptin-treated murine 4T1 BCa cells exhibited decreased VEGF and VEGF Receptor (VEGFR) mRNA and protein levels in the presence of mouse anti-IL1R antibody. The IKK/IKK inhibitor BAY11-7082 prevented the IL-1-NF- κ B-dependent upregulation of CXCR-7 in endothelial progenitor cells (EPCs), which is induced by C-X-C chemokine receptor type 7 (CXCR-7). Anakinra or IL-1Ra decreased tumor angiogenesis *in vivo*, while IL-1Ra suppressed the proliferation of the HUVEC endothelial cell line *in vitro* and the growth and vascularization of the MCF7 BCa xenograft tumor *in vivo*. Finally, mouse DA3 BCa tumor vascularization and development were reduced in IL-1

and IL-1 deletion animals (Voronov *et al.*, 2003b). According to the preceding discussion of HER2 and ER expression, four classifications were determined. 86% of HER2-positive but ER-negative malignancies had high levels of NF- κ B activation, compared to 33% of basal-like cancers (HER2-negative and ER-negative; 3/9) and only slightly in ER-positive cancers (K. J. Park *et al.*, 2005). HER2-positive, ER-negative breast tumors most definitely have NF κ B activation. Although we haven't particularly looked into this issue, it's possible that the NF- κ B pathway is activated in the portion of basal-like breast cancers that overexpress EGFR. It appears that ER-positive breast cancers specifically inhibit NF κ B activation, and it's possible that this inhibition is influenced by the amount of sex steroid ligands present. NF- κ B may also be involved in endocrine resistance, particularly to aromatase inhibitors, which rob the estrogen receptor of its preferred ligand. Although NF- κ B activation is variably regulated in several subclasses of breast cancers, its function in transformation or the onset of breast cancer has not been studied (Sørlie *et al.*, 2001). When the receptor tyrosine kinase ErbB2 is overexpressed in mammary epithelial cells, it results in the development of the disease and a sequential progression from hyperplasia to DCIS and IBS. We employed MMTV-ErbB2 mice. This mouse model accurately reproduces the progression of human breast cancer, especially the ErbB2/HER2 positive subtype. (Guy *et al.*, 1992) IL-1 has both cytotoxic and cytostatic effects, but it can also encourage the growth of cancer cells. Chronic inflammation brought on by local senescent fibroblasts can lead to tumor start and progression in the aging-related disease of cancer. Apigenin, an anti-inflammatory flavonoid, was demonstrated to diminish inflammatory cytokine secretion and paracrine activation of MDA-MB-231 TNBC and luminal ZR-75-1 BCa cell proliferation. Apigenin suppresses IL-1 and NF- κ B signaling in senescent fibroblasts. As a result, paracrine factors that encourage BCa proliferation are produced by IL-1-NF- κ B signaling in senescent fibroblasts. In order to test the idea that cytokines involved in bone remodeling, such as IL-1, can promote BCa proliferation in the bone, osteoblasts were co-cultured in 3D with either luminal A MCF7 cells or non-metastatic MDA-MB-231 TNBC subline cells. The BCa cells were inactive in the absence of IL-1, but they began to proliferate when IL-1 was added. These findings imply that IL-1 pro-tumorigenic

actions require a paracrine environment that is particular to the bone metastatic niche. While IL-1 can be anti-tumorigenic in the initial tumor, it can also increase osteoclast activity in bone, which can result in bigger osteolytic BCa lesions. All of these results point to the fact that IL-1 causes BCa tumor suppression or expansion that is particular to the context and is controlled by the characteristics of the tumor microenvironment (Tulotta *et al.*, 2019). Breast cancer in vivo, clinical biopsies, and breast cancer of rat, they discovered that 20 out of 23 samples tested positive for the c-Rel subunit using immunoblotting of nuclear extracts, indicating that nuclear localization of NF-kB/Rel expression is a feature of human breast cancer tissue. These findings, which demonstrate that NF-kB binding activity and concentration are both elevated in human breast cancer tissue, have been confirmed and expanded (Cogswell *et al.*, 2000). Studies on the NF-kB pathway in breast cancer conducted in vivo and in vitro are presented, with an emphasis on DNA damage and the epithelial-mesenchymal transition linked to breast cancer cell characteristics (Y. H. Park, 2017). The expression of the transcription factors involved in the epithelial-to-mesenchymal transition (EMTTFs) SNAIL, SLUG, SIP1, and TWIST1 is associated with the metastatic process in breast cancer. Nuclear factor-kappa B (NF-kB) activation and EMT-TFs have been linked to the aggressiveness and potential for metastasis in carcinomas. In BRCA1 mutation carriers, the receptor activator of NF-kB (RANK) and its ligand (RANKL) play significant roles in the development of breast cancer. BRCA1 mutation carriers have higher levels of ductal epithelial progenitor cells that are RANK-positive than healthy people. In BRCA1 mutation carriers, these RANK-positive progenitor cells are where breast cancer first developed (Nolan *et al.*, 2016). In accordance with reports, the BRCA2 promoter is activated in breast cancer cells by NF-kB. The reported overexpression of the BRCA2 tumor suppressor gene in breast cancers may be a result of constitutive NF-B expression and DNA binding. Therefore, altering the expression of BRCA2 may be one way whereby abnormally regulated NF-kB leads to oncogenesis (Sovak *et al.*, 1997).

Cytidine deaminase (AID), a mutator enzyme, is induced as another way for NF- κ B to promote tumor (Colotta *et al.*, 2009). The suppression of prostaglandin-endoperoxide synthase 1 (PTGS1; also known as COX1) and PTGS2 (COX2) is the main mechanism underlying its anticancer effects. Cell proliferation, angiogenesis, and resistance to apoptosis are all caused by COX1 and COX2, which support the production of prostaglandins from arachidonic acid and maintain inflammatory signals brought on by the NF- κ B pathway. Additionally, they trigger the Wnt-catenin pathway's activation, which aids in the growth and development of cancer. However, inflammatory mediators such cytokines, prostaglandins, reactive oxygen species (ROS), and reactive nitrogen species can cause genetic changes that lead to carcinogenesis when the inflammatory response persists (Grivennikov *et al.*, 2010a). By directly promoting the production of urokinase-type plasminogen activator (uPA), metalloproteinase-9 (MMP-9), and chemokine receptor CXCR4, NF- κ B increases extracellular matrix breakdown and subsequent breast cancer cell migration. Similarly, animals with I κ B alpha-deficient mammary epithelial cells transplanted into their mammary glands displayed less complete extracellular matrix (Sliva *et al.*, 2002). The regulation of additional antiapoptotic genes by NF- κ B may also have an impact on how well a patient responds to treatment. For instance, manganese superoxide dismutase (Mn-SOD) expression is increased when NF- κ B is activated. The free-radical scavenger Mn-SOD lessens the oxidative damage brought on by chemotherapy. Paclitaxel, hydrogen peroxide, and okadaic acid resistance have been shown to be correlated with high Mn-SOD expression levels in breast cancer cells. Furthermore, it has recently been proposed that radiation-induced adaptive responses are mediated via NF- κ B-mediated Mn-SOD production (Patel *et al.*, 2000). As an example, overexpressing I κ Balpha boosted paclitaxel-induced G2/M arrest and death in MDA-231 breast cancer cells. Additionally, these cells expressed less of the NF- κ B-controlled antiapoptotic genes XIAP and BclxL. Similarly, when I κ Balpha was overexpressed in MDA-231 cells, the number of cells that formed colonies was greatly reduced. When MDA-231 cells overexpressing I κ Balpha were injected into the mammary glands of mice, tumor growth was suppressed and the

number of metastatic foci was reduced. These data suggest that inhibiting NF- κ B in breast cancer cells may enhance the therapeutic effectiveness of paclitaxel by promoting apoptosis and reducing colony formation, tumor growth, and metastasis. The DNA-binding activity of NF- κ B is generally associated with poor outcomes in breast cancer patients (Dalmases *et al.*, 2014). The NF- κ B pathway and doxorubicin resistance in triple negative breast cancer (TNBC) have been linked, according to research from our lab. We carried out this research to determine how transcription factor NF- κ B family genes and their regulatory elements influence the response to adjuvant chemotherapy, including doxorubicin, in patients with TNBC. We discovered that the expression level of the SP1 gene was a potential prognostic marker in TNBC patients receiving adjuvant doxorubicin chemotherapy (J. Y. Kim *et al.*, 2016). Furthermore, in relation to the expression of APOBEC3, RANK signaling has become a significant potential target of the NF- κ B pathway. As a novel approach to treat resistant breast tumors, addressing the NF- κ B pathway in breast cancer has therapeutic potential and prospective benefits (Leonard *et al.*, 2015).

Targeting particular signaling pathways that promote abnormal cell growth and survival has become the primary goal of cancer treatment in recent years, with the promise of increased specificity and decreased systemic toxicity. In breast cancer cells, NF- κ B has been shown to up-regulate VEGF mRNA, suggesting its function in angiogenesis and the spread of breast cancer (D. W. Kim *et al.*, 2000). A useful indicator of a target or pathway's significance in the malignancy process and of the potential efficacy of a medication that acts on that target or pathway is the frequency with which it experiences mutation or deregulation. IKK/NF- κ B signaling module is a crucial signaling route that is frequently changed in human malignancies (Bassères & Baldwin, 2006). Recently they discovered prototypical and equivalent impacts on the course of the cell cycle and apoptosis when we used experimental manipulations that blocked several points on the stream events leading to NF- κ B activation, such as PI3K activity, phosphorylation of AKT, IKK activity, and NF- κ B activation. Numerous studies have demonstrated that NF- κ B is necessary for Ras-

mediated transformation and that oncogenic Ras increases NF- κ B-dependent transcription. Multiple lines of evidence show that NF- κ B and PI3K/Akt work together in a pathway that prevents TGF- β from inducing apoptosis in Ras-transformed mammary cells. For instance, PS1145 is a small molecule developed by Millennium Pharmaceuticals that inhibits IKK. This small molecule in the micromolar range, is capable of inhibiting IKK, and is inactive against a variety of other kinases, including src, MMEK, and the EGFR kinase, and it can inhibit IKK at micromolar concentrations (Weinstein, 2000). The usage of protease inhibitors boosted the lymphoma cells' apoptosis caused by radiation. Additionally, the use of particular NF- κ B pathway inhibitors has made the precise mechanisms underlying radiation-induced apoptosis more understandable. Intriguingly, the authors' institution is investigating the radio sensitizing effects of cyclooxygenase-2 inhibitors, whose therapeutic action has been demonstrated to utilize an NF- κ B-dependent route. The results thus far are encouraging. Response to chemotherapy and radiation and NF- κ B signaling in conventional cancer therapies. In cells treated with chemotherapeutic drugs such doxorubicin, paclitaxel, and topoisomerase inhibitors [and in cells exposed to radiation, which is used to treat breast cancer], NF- κ B activation has frequently been seen. Tamoxifen, raloxifene as well as the aromatase inhibitors exemestane and anastrozole are some of the hormonal treatments advised by international guidelines to mitigate the risk of local recurrence or tumor development in contralateral breast tissues after surgery. According to reports, these medications lessen the risk of hormone receptor-positive breast cancer. Phase I/II study of bortezomib and capecitabine in patients with metastatic breast cancer found a moderate antitumor effect in heavily pretreated patients, breast cancer patients with an activated NF- κ B pathway might benefit from bortezomib treatment. Approximately 20% of human breast tumors that respond to trastuzumab and newer small molecule receptor tyrosine kinase inhibitors have significant levels of HER2 amplification and receptor expression which has significant effect in breast cancer treatment. The vast majority of node-negative people survive their disease without adjuvant therapy, while 20 to 30% die despite adjuvant systemic therapy. It has been estimated that chemotherapy only saves breast

cancer mortality in 10% of node-negative patients; around 70% of these patients do. Therefore, NF- κ B activation may inhibit apoptosis and boost metastasis, resulting in a more aggressive phenotype in breast cancer. In some node negative patients, early adjuvant therapy may be necessary to lessen the possibility for tumor cells to spread to other organs (Mulero *et al.*, 2019). Breast cancer is a heterogeneous disease which may influence the interaction of nuclear factor kappa b and levels of ER so further studies specific with nuclear factor kappa b we can bring an anticancer target for breast cancer

2.5 Tumor Necrosis factor- α in breast cancer

An important role for tumor necrosis factor alpha (TNF- α) has been identified in the intricate interactions across molecular pathways that drive the development and spread of breast cancer. Prior to first for its function in controlling inflammation and immunological responses, TNF- α 's connection to the development of breast cancer has attracted a lot of interest lately. Research has revealed its complex effects on angiogenesis, apoptosis, metastasis, and the tumor microenvironment, which in turn affect tumor aggressiveness and response to therapy. Interestingly, TNF- α plays two distinct functions in the biology of breast cancer, functioning as a tumor suppressor and promoter in response to different stimuli, treatment approaches, and tumor subtypes (Balkwill, 2002; Sethi *et al.*, 2008). Through its interaction with TNF receptors (TNFR1 and TNFR2), TNF- α mechanistically orchestrates complex signaling cascades that activate downstream pathways such as nuclear factor-kappa B (NF- κ B), Mitogen Activated Protein Kinases (MAPKs), and PI3K/AKT. These pathways collectively regulate cell survival, proliferation, and migration (Aggarwal, 2003; Kuprash & Nedospasov, 2016).

Moreover, new data indicates that TNF- α signaling and hormone receptor pathways interact, linking TNF- α to resistance to endocrine therapy and hormone driven tumor growth (Obradović *et al.*, 2019). Clinically, patients with breast cancer who have elevated TNF- α levels have been linked to a poor prognosis, an aggressive tumor, and resistance to standard therapy (Karin & Lin, 2002; Vargo-Gogola & Rosen, 2007). Thus, focusing on TNF- α and its

downstream effectors shows promise as a possible therapeutic approach to slow the advancement of breast cancer and enhance therapy results. To clarify its exact function in breast cancer pathogenesis and its treatment implications, more study is necessary due to the intricacy of TNF- α signaling networks and its context-dependent effects (Liu *et al.*, 2017; Tergaonkar, 2006).

2.6 IL-6 and IL-1 β in breast cancer

The development and progression of breast cancer are linked to two important pro-inflammatory cytokines: interleukin-6 (IL-6) and interleukin-1 beta (IL-1 β). Their complex functions go beyond immune regulation to include angiogenesis, invasion, proliferation, and metastasis, among other facets of tumor biology. An increasing body of research indicates that abnormalities in IL-1 β and IL-6 signaling play a role in the development, growth, and resistance to treatment of breast cancer (Grivennikov *et al.*, 2010b; Mantovani *et al.*, 2008). Breast cancer cells are subject to pleiotropic effects from IL-1 β , which is mainly produced by tumor-associated macrophages (TAMs) and cancer cells inside the tumor microenvironment. By means of its engagement with IL-1 receptors (IL-1Rs), namely IL-1R1, IL-1 β triggers subsequent signaling pathways, including NF- κ B and MAPKs, thereby endorsing tumor cell proliferation, motility, and infiltration (Apte *et al.*, 2006; Voronov *et al.*, 2003c).

Additionally, pro-angiogenic proteins including vascular endothelial growth factor (VEGF) are enhanced in expression by IL-1 β , which promotes tumor angiogenesis and metastasis (Elaraj *et al.*, 2006). The potential of increased IL-1 β as a prognostic biomarker and therapeutic target has been highlighted by clinical studies that have connected it with advanced disease stage, poor prognosis, and lower survival rates in breast cancer patients (Voronov *et al.*, 2014). In a comparable way, IL-6, which is mostly released by immune cells, fibroblasts, and adipocytes that infiltrate tumors, is essential to the pathophysiology of breast cancer. In order to cause the transcriptional upregulation of genes involved in cell proliferation, survival, and metastasis, IL-6 binds to the IL-6 receptor (IL-6R) and activates the Janus kinase/signal

transducer and activator of transcription (JAK/STAT) pathway (Knüpfers & Preiss, 2010; Sansone *et al.*, 2007). Furthermore, IL-6 promotes tumor aggressiveness and resistance to therapy by maintaining characteristics similar to cancer stem cells and the epithelial-mesenchymal transition (EMT) (Iliopoulos *et al.*, 2011; S. Y. Kim *et al.*, 2013). Research has shown that poor clinical outcomes, such as higher tumor grade, potential for metastasis, and treatment resistance in patients with breast cancer, are correlated with raised IL-6 levels (Bachelot *et al.*, 2003; Salgado *et al.*, 2003). Moreover, new research points to complex interactions between the IL-1 β and IL-6 signaling pathways, which enhances the former's pro-tumorigenic effects in breast cancer. A positive feedback loop that maintains chronic inflammation and tumor growth can be formed when IL-1 β stimulates the production of pro-inflammatory cytokines such as IL-6 (Apte & Voronov, 2002; Langowski *et al.*, 2006). On the contrary present, by increasing the expression of IL-1 β and its receptors, IL-6 can potentiate IL-1 β signaling and strengthen the inflammatory state inside the tumor microenvironment (Kishimoto, 2010; Yeh *et al.*, 2006). In order to effectively reduce tumor development and metastasis, preclinical studies have shown that targeting the IL-1 β /IL-6 axis is a promising therapeutic approach in the therapy of breast cancer (Lin & Karin, 2007; Rose-John, 2012). IL-1 β and IL-6 are essential for coordinating the inflammatory tumor microenvironment and promoting the advancement of breast cancer. Their significance as possible prognostic markers and therapeutic targets in the therapy of breast cancer is highlighted by their complex interactions with several signaling pathways.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To investigate the efficacy of linoleic acid in targeting breast cancer by analyzing its effects on the expression of inflammatory genes through both in vivo experimentation and in silico docking studies.

3.2 OBJECTIVES

1. Determine the impact of linoleic acid treatment on the expression levels of NF κ B, TNF- α , IL-1 β , and IL-6 in breast cancer.
2. Utilize computational docking simulations to predict the binding affinity of linoleic acid with key proteins involved in the inflammatory pathway (NF κ B, TNF- α , IL-1 β , and IL-6).
3. Identify potential binding sites and interaction patterns between linoleic acid and the target proteins through in silico analysis.
4. Correlate in vivo findings with in silico docking results to elucidate the structural basis of linoleic acid's anti-inflammatory effects in breast cancer.
5. Explore the therapeutic potential of linoleic acid as a modulator of inflammatory pathways in breast cancer, integrating insights from both experimental and computational approaches.
6. Validate the efficacy of linoleic acid as a targeted therapy for breast cancer through comprehensive in vivo and in silico assessments.

CHAPTER 4

MATERIALS AND METHODOLOGY

4.1 Materials

4.1.1 Compound Used

- Linoleic acid – Ligand
- PPAR γ – Protein

4.1.2 Software Used

- Molegro Molecular Viewer.
- PyRx Vina Auto dock.
- Igem Dock

4.1.3 Website Used

- PubChem
- Swissadme
- Protox II
- Chemical Entities of Biological Interest (ChEBI)
- SwissProt
- PDB (Protein Data Base)

4.2 *Insilico* methodology

4.2.1 *Linoleic acid* – ligand

Linoleic acid is a doubly unsaturated fatty acid, also known as an omega-6 fatty acid, occurring widely in plant glycosides. Linoleic acid (LA) is an organic compound with the formula $\text{HOOC}(\text{CH}_2)_7\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_4\text{CH}_3$. In this particular polyunsaturated fatty acid (PUFA), the first double bond is located between the sixth and seventh carbon atom from the methyl end of the fatty acid (n-6). It is a fatty acid sometimes denoted 18:2 (n-6) or 18:2 cis-9,12. It is a colorless liquid that is virtually insoluble in water but soluble in many organic solvents. It typically occurs in nature as a triglyceride (ester of glycerin) rather than as a free fatty acid. It is one of two essential fatty acids for humans, who must obtain it through their diet, and the most essential, because the body uses it as a base to make the

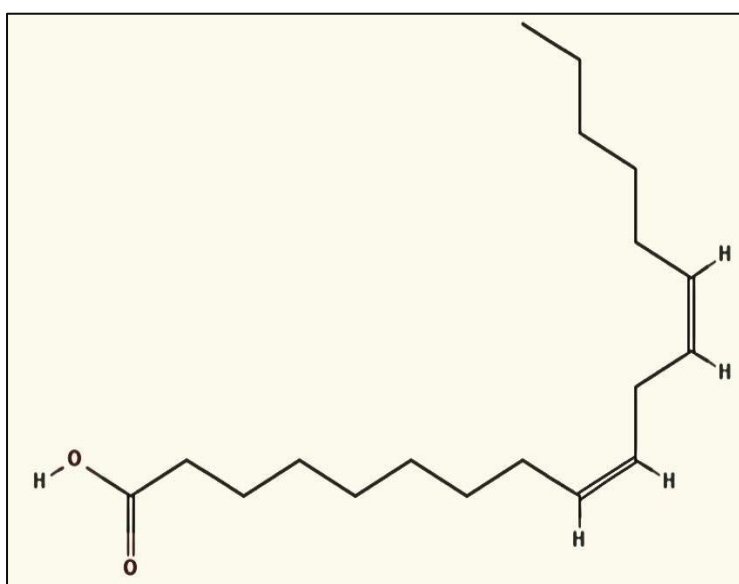


Figure 4.1 Structure of linoleic acid

PUBCHEM ID: 5280450

SMILES ID: CCCCC=CCC=CCCCCCCC(=O)O

4.2.2 Peroxisome Proliferator Activated Receptor γ – Protein

Peroxisome proliferator-activated receptor gamma (PPAR- γ or PPARG), also known as the glitazone reverse insulin resistance receptor, or NR1C3 (nuclear receptor subfamily 1, group C, member 3) is a type II nuclear receptor functioning as a transcription factor that in humans is encoded by the *PPARG* gene. PPARG is mainly present in adipose tissue, colon and macrophages. Two isoforms of PPARG are detected in the human and in the mouse: PPAR- γ 1 (found in nearly all tissues except muscle) and PPAR- γ 2 (mostly found in adipose tissue and the intestine)

PPARG regulates fatty acid storage and glucose metabolism. The genes activated by PPARG stimulate lipid uptake and adipogenesis by fat cells. Many naturally occurring agents directly bind with and activate PPAR gamma. These agents include various polyunsaturated fatty acids like arachidonic acid and arachidonic acid metabolites such as certain members of the 5-hydroxyicosatetraenoic acid and 5-oxoeicosatetraenoic acid family. The activation of PPAR gamma by these and other ligands may be responsible for inhibiting the growth of cultured human breast, gastric, lung, prostate and other cancer cell lines

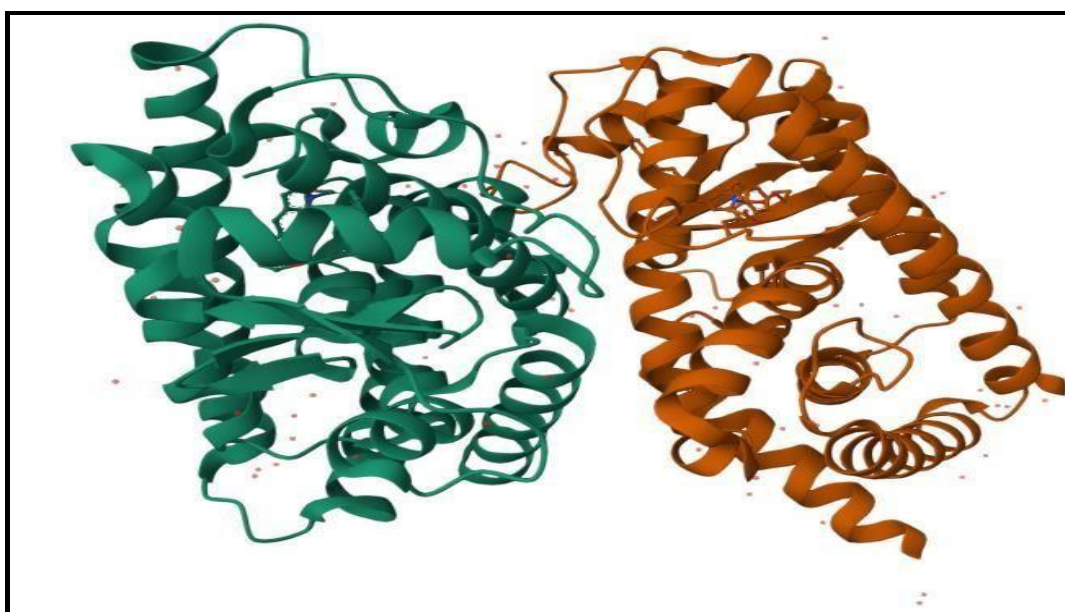


Figure 4.2: Structure of PPAR γ

4.2.3 Validation of ligand- Linoleic acid

4.2.4 Preparation of ligand

The three-dimensional structure of linoleic acid is extracted from drug bank in pdb format. To ensure the accuracy and reliability of the molecular representation, the structure underwent meticulous scrutiny using the Molegro software. This rigorous examination aimed to verify the absence of any extraneous molecules and to refine the molecular composition of linoleic acid, thereby ensuring the integrity of its structural conformation. By employing advanced computational techniques, including thorough ligand preparation and validation, this study aims to provide a comprehensive understanding of linoleic acid's molecular interactions and pharmacological properties. Such insights into the structural characteristics and functional roles of linoleic acid hold significant implications for its potential therapeutic applications in various disease contexts, ranging from cardiovascular disorders to inflammatory conditions.

4.2.5 Preparation of protein

The peroxisome proliferators-activated receptor- γ 's three-dimensional crystallographic structure (PDB ID: 3NOA) was obtained from the Protein Data Bank (www.rcsb.org). The PDB serves as a repository for structural data of biomolecules, facilitating research in understanding their roles in health and disease. By elucidating the intricate arrangement of atoms within PPAR- γ , researchers gain essential knowledge to uncover disease mechanisms and develop targeted therapeutic interventions. To gain a deeper understanding PPAR- γ 's structure-function relationship, a thorough analysis was conducted utilizing various computational tools and techniques. One such analysis involved examining the conformational preferences of amino acid residues through the construction of a Ramachandran plot. This plot allows visualization of the phi (ϕ) and psi (ψ) angles, providing insights into the stability and sterically allowed regions of the protein's backbone.

Additionally, employing the Schrödinger software's protein preparation wizard was instrumental in refining the initial PDB structure. This process involved the removal of crystallographic water molecules and the addition of hydrogen atoms, followed by optimization and minimization to ensure a physiologically relevant conformation. Subsequently, the structural integrity and quality of the protein were assessed using tools such as CastP, Procheck, and the Errat online server. CastP analysis facilitated the identification and characterization of potential protein pockets or binding sites within PPAR- γ , shedding light on its ligand-binding capabilities and potential drug targets. Procheck evaluation provided crucial insights into the overall stereochemical quality of the protein structure, ensuring it adheres to acceptable geometric parameters. Furthermore, utilizing the Errat online server allowed for the assessment of non-bonded atomic interactions and the overall reliability of the protein model. The comprehensive structural analysis of PPAR- γ using advanced computational tools not only enhances our understanding of its biological function but also provides a foundation for the rational design of therapeutic agents targeting this important nuclear receptor. Such insights hold promise for the development of novel treatments for a myriad of diseases, ranging from metabolic disorders to inflammatory conditions.

4.2.6 Docking

Docking is a computational method used in drug discovery to predict how a small molecule, typically a drug candidate, will bind to a target protein. The goal of docking is to predict the most energetically favorable binding orientation of the ligand within the binding pocket of the protein.

The basic idea behind docking is to predict the best orientation of a tiny molecule within a target protein's binding site. The procedure entails producing small molecule conformations and calculating the energy of interaction between the ligand and the protein. The projected binding pose is then chosen based on the tiny molecule's most energetically favorable conformation. Docking consists of four basic steps: target preparation, which

includes downloading the target protein in 3D format and removing unnecessary elements like water. Ligand is currently being downloaded in SDF format. The binding location is then defined and edited. Docking is handled by Lib doc, a module in Discovery Studio that employs rigid body docking techniques. The results are then analyzed based on the binding site and ligand affinity for the target protein. Docking is frequently used to identify prospective drug candidates as a first stage in the drug discovery process. It can also be used to improve existing pharmaceuticals or to discover new applications for existing drugs.

4.2.7 Validation docking

Validation docking stands as a pivotal step in computational biology and drug discovery, dedicated to scrutinizing the accuracy and reliability of protein-ligand interaction predictions generated by docking algorithms. In this realm, iGem Dock emerges as a notable tool, meticulously crafted for protein-ligand docking simulations. Developed by the International Genetically Engineered Machine (iGEM) Foundation, iGem Dock harnesses sophisticated algorithms to forecast the binding modes and affinities between proteins and small molecules. Validation docking entails subjecting these predictions to rigorous testing against experimental data, ensuring the computational models faithfully portray real-world interactions. Through the utilization of validation docking, researchers can evaluate the performance of docking algorithms, validate predictions against experimental outcomes, and glean insights into the strengths and limitations of computational methods in drug discovery. This iterative process is indispensable for refining computational models, guiding experimental design, and ultimately expediting the development of novel therapeutics with augmented efficacy and safety profiles.

4.3 *Invitro* methodology

4.3.1 Cell culture

The cell lines (C2C12 and MDA-MB-231) were purchased from National Centre for Cell Science (NCCS), Pune and were grown in DMEM, supplemented with 10% FBS and were maintained in a humidified atmosphere containing 95% air and 5% CO₂ at 37 °C. In a culture vessel, the cells are grown to confluence, generating a monolayer that adheres to the surface. Next, additional cells are sub cultured by the use of the trypsinization process. The culture medium is then aspirated, and 2ml of phosphate buffered saline (PBS) are used to rinse the cells in order to get rid of any remaining medium and cellular debris. Add 1 ml of 0.05% trypsin, and let it trypsinate for three to five minutes at 37°C. To see the cells separate, gently tap the side of the dish; if this is not done carefully, over-trypsinization may happen. To neutralize the trypsin, 2 ml of medium is then added. After three minutes of room temperature spun at 900 rpm, the cell suspension is aspirated to remove the supernatant. After collection, the pellet was reconstituted in 2 ml of DMEM medium containing 10% FBS. After that, the cells were divided into four sterile, clean bottles, each holding a volume of 0.5 ml. The bottles were then filled to a capacity of 2 ml with DMEM media.

Table 4.1 List of Cell Lines used

Type of Cell line	Cell line	Medium used	Source of cell line
Control breast cells	C2C12 - Normal Fibroblast cells	DMEM with 10% FBS	National Centre for Cell Science (NCCS), Pune
Breast Cancer cells	MDA-MB-231	DMEM with 10% FBS	National Centre for Cell Science (NCCS), Pune

4.3.2 Preparation of the Drug

To prepare the drug solution, 10 microliters of linoleic acid were carefully measured and added to a solution of DMEM medium. The mixture was then subjected to vortexing for 5 minutes to ensure thorough mixing and dissolution of the drug with the medium

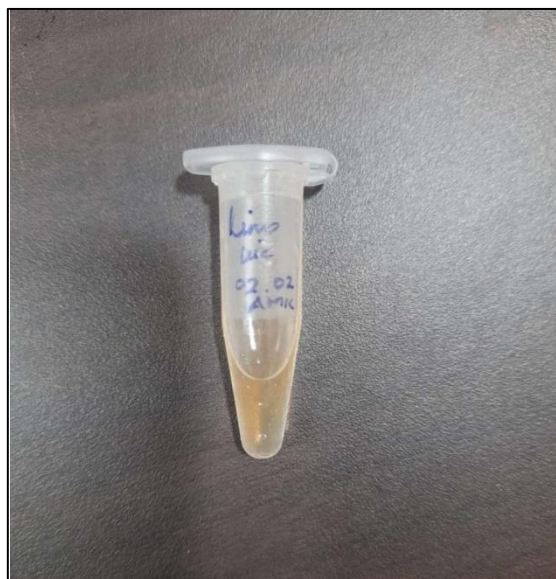


Figure 4.3: Dissolved Drug

4.3.3 Cytotoxicity Assay

Cell metabolic activity is measured using the MTT assay, which serves as indicators for cell viability, proliferation, and cytotoxicity. A yellow tetrazolium salt (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) is reduced to purple formazan crystals by metabolically active cells, which is the basis for this colorimetric assay. Enzymes known as NAD(P)H-dependent oxidoreductases are present in live cells, and they convert MTT to formazan. A solubilization solution is used to dissolve the insoluble formazan crystals, and a multi-well spectrophotometer is used to measure the color of the resultant-colored solution by measuring absorbance at 500–600 nanometres. The amount of live, metabolically active cells increases with solution darkness.

In Normal Cell line (C2C12)

Cell viability assays were performed in 96-well plates. MDA-MB-231 cells were cultured in the 96 plates until confluence and were incubated in a humidified incubator for 24 hours at 37°C. After 24 hours, cell confluency was checked using an inverted fluorescent microscope, and then various concentrations of the prepared drug, such as 10µl, 20µl, 30µl, 40µl, 50µl, 60µl, and 70µl, were added to 96 plates and incubated in a humidified incubator for another 24 hours. After 24 hours, the media from the cell cultures was discarded and serum-free media was added. A 96-well plate is now prepared for MTT addition. MTT salt was added 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. 50 µl of MTT is added to each well and incubated for 3 hours at 37°C. After incubation for 3 hours, serum-free media and MTT were removed. Add 150 µL of DMSO (dimethyl sulfoxide) 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 an OD of 570 nm.

In Cancer Cell line (MDA-MB-231)

Cell viability assays were performed in 96-well plates. MDA-MB-231 cells were cultured in the 96 plates until confluence and were incubated in a humidified incubator for 24 hours at 37°C. After 24 hours, cell confluency was checked using an inverted fluorescent microscope, and then various concentrations of the prepared drug, such as 10µl, 20µl, 30µl, 40µl, 50µl, 60µl, and 70µl, were added to 96 plates and incubated in a humidified incubator for another 24 hours. After 24 hours, the media from the cell cultures was discarded and serum-free media was added. A 96-well plate is now prepared for MTT addition. MTT salt was added 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. 50 µl of MTT is added to each well and incubated for 3 hours at 37°C. After incubation for 3 hours, serum-free media and MTT were removed. Add

150 μ L of DMSO (dimethyl sulfoxide) 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 an OD of 570 nm.

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



Figure 4.4: 96 well plate used for MTT assay

4.3.4 Acridine Orange/Ethidium Bromide (AO/EB) Fluorescent Staining

Fluorescent dyes like Acridine Orange (AO) and Ethidium Bromide (EB) are frequently employed in cellular staining procedures, especially in cancer research. When attached to double-stranded DNA, the metachromatic dye AO, which can stain both living and dead cells, releases a green fluorescence. Conversely, EB only enters cells through broken membranes, intercalates DNA, and releases red fluorescence. To differentiate between necrotic, apoptotic, and living cells within a cell population, AO/EB staining is used. Apoptotic cells glow orange when AO penetrates the broken DNA, but living cells color green because AO binds to undamaged DNA. Red fluorescence is seen in dead cells, especially those that are necrotic, because EB binds to DNA in damaged membrane-bound cells. The staining of AO/EB on linoleic acid-treated and untreated cancer cells enables a comparison of the drug's effects on cellular viability and death mechanisms. Researchers can examine differences in the number of necrotic, apoptotic, and viable cells by staining the treated and untreated groups separately. This allows researchers to gain

insight into how well linoleic acid affects cancer cell viability and induces cell death.

MDA-MB-231 cells were cultivated on 6-well plates. 3 μ L of cell culture medium (DMEM) is added in each well. To promote the cells to stick together and grow, the plates were thereafter kept at 37°C for 24 hours in a humidified incubator. After the first incubation period of 24 hours, one set of wells (the "Treated group") received the drug concentration at an IC₅₀ value, while the other set of wells (the "Control group") stayed untreated. For evaluating the drug's impact on the cells, this step is vital. At a concentration of 10 mg/mL each of them stock solutions of ethidium bromide (EB) and acridine orange (AO) were made in PBS solution. After diluting 1 μ L of stock solution with 100 μ L of PBS, the final concentration of 1 mg/mL was achieved for both AO and EB. Fluorescence microscopy frequently uses these stains to observe different aspects of cell viability and morphology. Following the treatment phase, AO and EB were used to dye the cells. In Particular:

- In Control 1 and Treated 1 wells, only AO stain was added.
- In Control 2 and Treated 2 wells, only EB stain was added.
- In Control 3 and Treated 3 wells, a combination of both AO and EB stains (AO/EB stain) was added. Different cellular features can be seen under fluorescence microscopy due to this differential staining. To enable the dyes to enter the cells and attach to certain cell components, the stain solutions were kept in the wells for 20 minutes. After the staining time, an inverted fluorescent microscope was used to view the cells.

4.3.5 Total RNA Isolation

In cancer biology research, RNA separation is an essential step, especially for investigations involving gene expression analysis like PCR (Polymerase Chain Reaction). In order to study the transcriptional activity of genes inside the

biological sample, it entails the extraction of RNA molecules from cells or tissues. Gene expression levels are capable of being measured by means of RNA isolation. Researchers can determine how physiological factors or experimental treatments affect a gene's expression by analyzing the quantity of a particular RNA transcript. This knowledge is essential for figuring out possible treatment targets for illnesses like cancer as well as for comprehending the molecular mechanics underlying biological processes.

Furthermore, RNA isolation is necessary for subsequent processes like real-time PCR (qPCR) and reverse transcription PCR (RT-PCR), which are frequently used methods to assess the levels of gene expression. For these PCR-based techniques to produce reliable and precise outcomes, high-quality RNA templates must be provided. In order to reduce experimental variability and incorrect findings, accurate RNA isolation ensures the integrity and purity of the RNA samples.

MDA-MB-231 cells were cultivated on 6-well plates. 3 μ L of cell culture medium (DMEM) is added to each well. To encourage the cells to stick together and grow, the plates were kept at 37°C for 24 hours in a humidified incubator. After the first incubation period of 24 hours, the drug concentration at an IC₅₀ value was added to wells and incubated for another 24 hours in a humidified incubator at 37°C. The cells were rinsed the following day with an ice-cold PBS solution. Cells are scrapped after adding 1 ml of trizol, transferred to the Eppendorf, and kept at room temperature for 5 minutes. 250 μ L chloroform was added, shake vigorously for 15 seconds, and kept at room temperature for 5 minutes. The cells are centrifuged at 10,000 rpm for 5 minutes. After the centrifugation, three layers were obtained. The aqueous phase (first clear layer) was then pipetted out without disturbing the contents, transferred to a new 1.5 mL tube, and stored on ice. 550 μ L of isopropanol was added to the Eppendorf, which contained the aqueous phase, and left for 5 minutes at room temperature. Centrifuge it at 14,000 rpm for 20 minutes at 4°C, and keep it on ice carefully without shaking. Isopropanol was removed, and the pellet was washed using 75% ethanol and nuclease-free water. The

dried pellet was then resuspended in 25 μ L 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 μ L was also taken. The RNA was then kept at -20°C until used.

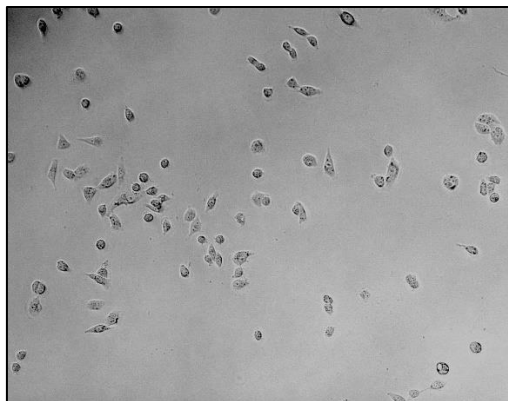


Figure 4.5: Cells treated with linoleic acid for RNA isolation



Figure 4.6: RNA isolation

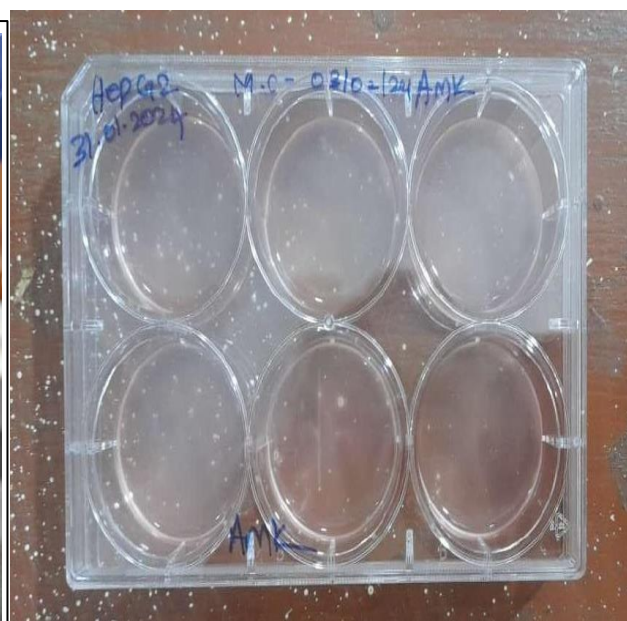


Figure 4.7: 6 well plates used for RNA

isolation

4.3.6 Agarose Gel Electrophoresis

A vital technique in molecular biology, agarose gel electrophoresis forms the basis for the examination of nucleic acids such as RNA. When separating the RNA from cancer cells that have been treated with linoleic acid, agarose gel electrophoresis is crucial. First off, it makes it possible to evaluate the integrity of the RNA, which is essential for ensuring the accuracy of subsequent molecular analyses. By observing discrete bands, biologists may determine the quality of extracted RNA and differentiate between undamaged and intact molecules. Second, the method makes it easier to estimate the amount of RNA, which helps assess whether enough RNA has been isolated for further tests such as reverse transcription PCR.

Agarose gel electrophoresis also acts as a detective tool, allowing impurities that might prevent precise molecular analysis to be found. Additional bands or smearing on the gel indicate the presence of any undesired materials, such as genomic DNA or leftover chemicals, indicating the need for further purification treatments. Methodologically, the procedure entails setting up an agarose gel, adding a tracking dye to RNA samples, electrophoresing them, and then using staining chemicals and UV light to visualize the separated RNA bands. In the final analysis, agarose gel electrophoresis plays a vital role in molecular biology research by offering information on the quantity, purity, and integrity of RNA that is essential for comprehending the molecular mechanisms behind treatments such as linoleic acid on cancer cells.

The first step in the gel electrophoresis procedure is making a 1x TBE (TrisBorate-EDTA) Electrophoresis Buffer. This is done by diluting the TBE stock solution with nuclease free water. To keep the environment sterile and avoid contaminating the samples, it is essential to thoroughly clean all instruments and equipment with nuclease free water. After dissolving agarose in the 1x TBE buffer, EtBr is added as a tracking dye to create a 1.2% agarose gel. This dye Ethidium bromide was added to the agarose gel. Some size markers may require significantly more than

10 µg/ml ethidium bromide for visualization. To accurately size your RNA, however, it is important to use the same amount of ethidium bromide in all the samples (including the size marker) because ethidium bromide concentration affects RNA migration in agarose gels. After that, the agarose solution is transferred into a gel mold and let to solidify. After the gel solidifies, the comb is carefully taken out, and the gel can be placed in the electrophoresis tank, submerged in 1x TBE buffer.

Following preparation by mixing with gel-loading buffer, 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 loaded into gel wells. Samples must be loaded gradually in order to prevent the introduction of air bubbles. The gel equipment is equipped with electrical leads, which facilitate the migration of RNA molecules towards the anode when a voltage is applied. The materials are subjected to electrophoresis until they migrate the desired distance, which is usually shown by the migration of bromophenol blue dye. The leads are withdrawn and the power supply is shut off after electrophoresis. After that, the gel is placed under an ultraviolet (UV) transilluminator so that the RNA bands may be seen. This is made possible by the fluorescent bromophenol blue dye. The results of the electrophoresis process, including the separation of RNA molecules based on size, are documented by photographing the gel under UV light. Throughout the procedure, meticulous attention to detail and adherence to proper technique are essential to ensure accurate results and minimize potential sources of error.

4.3.7 cDNA Conversion

One of the most important processes in molecular biology is the conversion of RNA into complementary DNA (cDNA), which allows for the use of cDNA in cloning and other downstream applications like gene expression research. To guarantee the integrity and caliber of the RNA template, this procedure usually starts with the isolation and purification of RNA from the

intended biological sample. By attaching themselves to the poly(A) tail of mRNA molecules, oligo(dT) primers are frequently used to start the reverse transcription step. Reverse transcriptase uses this hybrid primer-template to begin the process of synthesizing single-stranded cDNA by employing the RNA template. The reverse transcriptase enzyme is able to catalyze the conversion of RNA into cDNA when the reverse transcription reaction is conducted under carefully regulated circumstances, typically at a temperature of 37 to 42°C. The reaction is stopped after the proper incubation time, usually by heating the enzyme to inactivation. It is possible to guarantee the purity of the cDNA result by employing RNA-specific enzymes to eliminate any remaining RNA.

Using the iScript™ cDNA Synthesis Kit, 100 ng of total RNA was transformed to cDNA in two steps as per the manufacturer's instructions. Following the technique, 15µL of total RNA, 4µL of the master mix, and 1µL of the reverse transcriptase (RT) enzyme were combined to create a 20µL volume. The tubes underwent an incubation period of five minutes at 25°C for priming, 46°C for RT enzyme activation, one minute at 95°C for reverse transcriptase inactivation, and then four minutes at 4°C. To be used later, the tubes were kept in storage at -20°C.

4.3.8 Primer Designing

Primer designing using Euro genomics for genes such as NF-κB, IL-6, IL-1β and TNF-α involves inputting their sequences into the platform, which then utilizes algorithms to identify suitable primer binding regions. Multiple primer pairs are generated for each gene, ensuring specificity and efficiency. In silico analysis is performed to assess primer specificity and predict annealing temperatures. A comprehensive report detailing primer properties is provided, enabling researchers to select the most suitable primer pairs for experimental validation based on factors like specificity and efficiency. Overall, Eurogenomics streamlines primer design, facilitating the generation of high-quality primers tailored to the specific needs of molecular biology research



Figure 4.8: Dissolved primers

Table 4.2: List of Primers used for qRT-PCR

S.NO	TARGET GENE	GENE ID	FORWARD PRIMER	REVERSE PRIMER	AMPLICON LENGTH (bp)	Ta (°C)
1.	NF-κB	4791	CGAATGGA CAAGACAG CAG	CCGGAACAC AATGGCATA C	177	53.7
2.	IL-6	3569	ATAACCACC CCTGACCC CAAC	ATACCTCAAA CTCCAAAAG ACC	149	55.1
3.	IL-1β	7124	CTCTCACCT CTCCTACTC AC	ACACTGCTA CTTCTTGCC C	194	54.3
4.	TNF- α	3553	AACCATTCT CCTTCTCCC C	TTTCACCTCC ATCCATCCT C	186	51.3

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

Using quantitative real-time polymerase chain reaction (qRT-PCR), the gene expression levels of TNFα, IL6, IL1β, and NF-κB were examined. For

each target gene, gene-specific primers were used in accordance with the guidelines supplied by the SensiFAST™ SYBR® Lo-ROX Kit from Bioline, UK. To assure accuracy, duplicate analyses were performed on each sample. 5µL of SYBR Green master mix, 1.5µL of forward and reverse primers, and 3µL of cDNA template (50 ng) were combined for the qRT-PCR setup. The volume was then adjusted to 10µL using nuclease-free water. Using the Mastercycler Nexus Gradient Thermal Cycler from Eppendorf, Germany, PCR amplification was performed under the following thermal cycling conditions: 2 minutes of initial polymerase activation at 95°C, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60 to 65°C for 10 sec, and extension at 72°C for 15 sec. For 40 cycles, the cycle threshold value (Ct value) has been determined to measure the levels of gene expression. The $2^{-\Delta\Delta C_t}$ procedure was utilized for relative quantification, with the assumption that the reference sample had a relative gene expression of 1. The results were expressed as an expression fold change relative to the control, where a fold change of more than 1.0 indicated upregulation of the gene and less than 1.0 indicated downregulation. This thorough qRT-PCR study offers insightful information on the NF-κB, IL6, IL1β, and TNFα gene expression patterns under the experimental circumstances.



Figure 4.9: SYBR Green Super Super mix

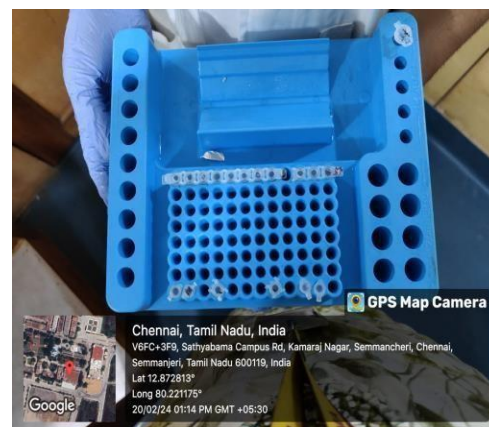


Figure 4.10: Samples after adding supermix

4.3.10 Statistical Analysis

An essential aspect of biological research is statistical analysis, which offers quantitative techniques for obtaining interpretation and conclusions from experimental data. In simple terms, statistical analysis is the process of using mathematical methods to examine and understand data in order to assist researchers in finding trends, patterns, and important distinctions between experimental conditions or groups.

Statistical analysis is essential in biological investigations for a number of reasons. First of all, it enables researchers to figure out if observed variations throughout experimental groups are merely the result of chance or statistically significant. This makes it easier to verify experimental results and derive trustworthy inferences from the information. Furthermore, statistical analysis makes it possible to compare experimental results with pre-established controls or standards, which makes it easier to assess experimental results in context with well-known biological processes. A popular kind of software used for statistical analysis and graphing scientific data is called GraphPad Prism. For researchers in the biological sciences, it provides an extensive set of statistical tests and graphing possibilities with a straightforward user interface. The unpaired t-test is a frequently used statistical test in biological experimentation. To find out if there is a significant difference between the means of two independent groups, this test is used to compare them. The t-test evaluates, accounting for sample size and group variability, whether the observed mean difference is greater than that which would be predicted by chance alone. A particular kind of unpaired t-test called the student t-test is applied in situations where sample sizes are limited and population standard deviations are not known. It computes the t-statistic, which is a measure of the variability within the groups divided by the difference between the means of the two groups. The chi-square test is another frequently utilized statistical tool in biological experimentation. The purpose of this test is to find out whether two categorical variables significantly correlate with one another. Assuming that

there is no correlation between the variables, it assesses whether the observed distribution of data deviates from the predicted distribution

5.1 VALIDATION OF LIGAND-RESULTS

Table 5.1: General characteristics of Linoleic acid

Linoleic acid (LA), identified by the PubChem ID 5280450, is a crucial polyunsaturated fatty acid. The SMILES ID CCCCC=CCC=CCCCCCCC(=O)O represents the molecular structure of linoleic acid in a text-based format (Simopoulos, 1999). This code can be used to visualize the molecule's two double bonds, a structural feature essential for its classification as a polyunsaturated fatty acid. The presence of two double bonds within the carbon chain qualifies linoleic acid as polyunsaturated. These double bonds influence the physical and chemical properties of linoleic acid, making it less saturated than saturated fatty acids and contributing to its liquid state at room temperature.

Table 5.2: Lipophilicity of Linoleic acid:

S. No	iLOGP	XLOGP3	WLOGP	MLOGP	SILICOS-IT	Consensus Log P _{o/w}
1.	4.14	6.98	5.88	4.47	5.77	5.45

In this table, various measures of the octanol-water partition coefficient (log P) for linoleic acid are presented. Log P is a crucial parameter in pharmacology and drug design as it quantifies the lipophilicity of a compound, which is a key determinant of its absorption, distribution, metabolism, and excretion (ADME) properties (Gleeson *et al.*, 2011). The different log P values reported here - iLOGP, XLOGP3, WLOGP, MLOGP, and SILICOS-IT - are calculated using different computational methods or algorithms. iLOGP represents the traditional log P value, while XLOGP3, WLOGP, and MLOGP are variations of log P calculated using different algorithms. SILICOS-IT, on the other hand, is a software tool that predicts log P values based on molecular descriptors. The Consensus Log P represents an average or consensus of these different log P values, providing a more robust estimation. For linoleic acid, these log P values range from 4.14 to 6.98, indicating its moderate to high lipophilicity. This suggests that linoleic acid is likely to have good membrane permeability and bioavailability, which are desirable properties for a potential drug candidate (Congreve *et al.*, 2003). Understanding these log P values is essential in drug design and formulation, as they influence the pharmacokinetic behavior and overall efficacy of a drug molecule.

5.1.3 Water solubility of phytoconstituents of linoleic acid

Table 5.3: Water solubility of linoleic acid

ESOL				Ali				SILICOS-IT			
Log S	Solubility		Class	Log S	Solubility		Class	Log S	Solubility		Class
	mg/mL	mol/L			mg/mL	mol/L			mg/mL	mol/L	
-5.05	2.49e-03	8.87e-06	Moderately soluble	-7.58	7.42e-06	2.64e-08	Poorly soluble	-4.67	5.93e-03	2.11e-05	Moderately soluble

The table provides crucial data on the solubility of linoleic acid, a significant fatty acid, as determined by different computational methods: ESOL, Ali, and SILICOS-IT.

Solubility is a vital property in drug design and formulation, directly impacting drug bioavailability and efficacy. In the ESOL column, the logarithm of the solubility (Log S) of linoleic acid is reported as -5.05, indicating a solubility of 2.49×10^{-3} mg/mL or 8.87×10^{-6} mol/L. This places linoleic acid in the category of moderately soluble compounds. The Ali method yields a Log S value of -7.58, corresponding to a solubility of 7.42×10^{-6} mg/mL or 2.64×10^{-8} mol/L, classifying linoleic acid as poorly soluble. SILICOS-IT, another computational tool, predicts a Log S of 4.67, suggesting a solubility of 5.93×10^{-3} mg/mL or 2.11×10^{-5} mol/L, indicating moderate solubility. It's evident that there are discrepancies between the predictions of different methods, which highlights the importance of considering multiple sources of data in drug development. Despite the variations, the data collectively suggest that linoleic acid exhibits moderate to poor solubility, underscoring the challenges that might arise in formulating drugs containing this compound. Therefore, in drug design activities, strategies to enhance the solubility of linoleic acid or its derivatives should be carefully considered to optimize its therapeutic potential.

5.1.4 Pharmacokinetic parameters of Linoleic acid:

Table 5.4: Pharmacokinetic parameters of Linoleic acid

GI absorption	BBB permeant	P-gp Substrate	CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4	Log Kp (cm/s)
High	YES	NO	YES	No	YES	NO	NO	-3.05cm/s

The pharmacokinetic parameters provided for linoleic acid are crucial indicators of its behavior within the body and its potential as a therapeutic agent. Linoleic acid is observed to have high gastrointestinal (GI) absorption, suggesting it can be efficiently absorbed from the digestive tract into the bloodstream (Cruciani *et al.*, 2000). Additionally, it is noted as a blood-brain barrier (BBB) permeant, implying its ability to cross the BBB and potentially exert effects within the central nervous system. However, linoleic acid is classified as a substrate for P-glycoprotein (P-gp), a transporter protein involved in efflux mechanisms that can limit the absorption and distribution of drugs. Furthermore, its interactions with cytochrome P450 enzymes (CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4) indicate its potential involvement in drug metabolism processes. Linoleic acid is metabolized by some of these enzymes, while others are not involved in its metabolism. Notably, CYP3A4, a key enzyme in drug metabolism, is identified as metabolizing linoleic acid. The Log Kp value of -3.05 cm/s suggests that linoleic acid has a relatively low permeability coefficient, which could influence its distribution and elimination from various tissues (Klopman *et al.*, 1994). Understanding these pharmacokinetic parameters is vital for elucidating the behavior of linoleic acid within the body, guiding its development as a therapeutic agent, and predicting potential drug interactions and adverse effects.

5.1.5 Drug Likeness of the Phytoconstituents of Linoleic acid:

Table 5.5: Drug Likeness of Linoleic acid

Lipinski	Ghose	Veber	Egan	Muegge	Bioavailability Score
YES	NO	NO	NO	NO	0.85

The table provides insights into the drug-likeness and bioavailability of linoleic acid, a significant fatty acid with potential therapeutic applications. According to Lipinski's Rule of Five, which assesses the drug-likeness of

compounds based on parameters like molecular weight, lipophilicity, and hydrogen bonding, linoleic acid meets the criteria, as indicated by the "YES" entry. This suggests that linoleic acid has properties favorable for oral absorption and potential as a drug candidate. However, it is notable that linoleic acid does not conform to Ghose's Rule, Veber's Rule, Egan's Rule, or Muegge's Rule, as indicated by the "NO" entries. Ghose's Rule evaluates molecular weight and lipophilicity, Veber's Rule assesses rotatable bonds, Egan's Rule considers polar surface area, and Muegge's Rule evaluates the distribution of lipophilic and hydrophilic moieties. The absence of adherence to these rules might suggest potential limitations in bioavailability or pharmacokinetic properties. Additionally, the bioavailability score of 0.85 indicates a moderate level of confidence in the oral bioavailability of linoleic acid, with room for improvement. Overall, while linoleic acid exhibits promising drug-like properties according to Lipinski's Rule, its lack of compliance with other rules and moderate bioavailability score suggest the need for further optimization and investigation to enhance its potential as a therapeutic agent.

5.1.6 Medical chemistry properties of phytoconstituents of Linoleic acid:

Table 5.6: Medical chemistry properties of Linoleic acid

Pains	Brenk	Lead likeness	Synthetic accessibility
0 alert	1 alert	No; 2 violations: MW>350	3.10

The medical chemistry properties of phytoconstituents of linoleic acid are critical indicators of their suitability for drug development and medicinal applications. According to the PAINS (Pan-Assay Interference compounds) filter developed by Brenk *et al.*, linoleic acid's phytoconstituents do not trigger any alerts, suggesting they do not exhibit common structural motifs associated

with assay interference or false-positive results in high-throughput screening assays. This is a positive indication of the potential utility of linoleic acid derivatives in drug discovery efforts, as compounds that pass this filter are less likely to produce misleading results during early-stage screening campaigns (Tetko *et al.*, 2005). Additionally, the lead likeness assessment indicates that one alert is triggered, suggesting that the phytoconstituents of linoleic acid possess structural features similar to those found in known lead compounds. This can be advantageous in identifying bioactive molecules or scaffolds for further optimization in drug discovery programs. Moreover, the synthetic accessibility assessment reveals that there are no violations in terms of synthetic accessibility, except for two violations where the molecular weight exceeds 350 . This indicates that while the synthesis of linoleic acid derivatives might be feasible, there may be challenges associated with compounds with molecular weights above 350. Overall, these medical chemistry properties suggest that linoleic acid and its derivatives hold promise as potential drug candidates, with favorable characteristics for medicinal chemistry optimization and further development

5.1.7 Toxicity of Linoleic acid:

Table 5.7: Toxicity of Linoleic acid

Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity	AhR	AR	AR-LBD	Aromatase	ER	ER-LBD	PPAR- γ	nrf2/ARE	HSE	MAP	p53	ATAD6
yes	no	yes	no	no	no	no	no	yes	yes	yes	no	no	no	no	no	no

The toxicity profile of linoleic acid is a crucial aspect to consider in evaluating its safety for therapeutic and nutritional applications. According to the provided data, linoleic acid is predicted to exhibit hepatotoxicity and immunogenicity, as indicated by the "yes" entries in the corresponding columns. Hepatotoxicity

refers to the potential of a substance to cause liver damage, which is a significant concern in drug development and dietary supplementation. Immunogenicity suggests that linoleic acid may stimulate the immune system, potentially leading to adverse immune reactions(Ciallella & Zhu, 2019).

On the other hand, linoleic acid is predicted to not be carcinogenic, mutagenic, or cytotoxic, as indicated by the "NO" entries in these columns. This indicates that linoleic acid is unlikely to induce cancer, genetic mutations, or cell death under the conditions evaluated. However, it's important to note that toxicity predictions are based on computational models and should be interpreted with caution, as they may not fully capture the complex interactions and physiological responses in living organisms. Nevertheless, these predictions provide valuable insights into the potential safety profile of linoleic acid and highlight areas of concern that warrant further investigation, particularly in preclinical and clinical studies. Overall, understanding the toxicity profile of linoleic acid is essential for ensuring its safe use in various applications, including pharmaceuticals, dietary supplements, and functional foods.

5.2 Docking Results

The results coincides with the program outcome for the ability to use modern tools

Linoleic acid was docked with PPAR γ by using the PyRx docking software. The docked results were analyzed using the Root Mean Square Deviation and the binding affinity. The lower the RMSD and higher the negative value of binding affinity which indicates the better agreement between the docked pose and the crystal structure. The validation was carried out by iGem dock. The PPAR γ was docked with linoleic acid compounds and the results output was delivered by the means of Hydrogen bonding and the Vander Waal's cut off and the interacting amino acids.

Table 5.8: Docking Results from PyRx

S. No	Ligand	Binding Affinity	rmsd/ub	rmsd/lb
1.	processed_ligand_linoleic_acid_uff_E=43.86	-4.3	0	0
2.	processed_ligand_linoleic_acid_uff_E=43.86	-4.2	8.587	5.808
3.	processed_ligand_linoleic_acid_uff_E=43.86	-4.2	8.523	5.679
4.	processed_ligand_linoleic_acid_uff_E=43.86	-4.2	2.694	2.248
5.	processed_ligand_linoleic_acid_uff_E=43.86	-4.1	12.475	10.356
6.	processed_ligand_linoleic_acid_uff_E=43.86	-4	3.569	2.653
7.	processed_ligand_linoleic_acid_uff_E=43.86	-4	6.533	2.697
8.	processed_ligand_linoleic_acid_uff_E=43.86	-3.8	9.969	6.195
9.	processed_ligand_linoleic_acid_uff_E=43.86	-3.7	13.516	10.985

The docking results obtained for PPAR gamma with linoleic acid reveal crucial insights into the interaction between the receptor and the ligand. Among the entries in the table, varying binding affinities were observed, ranging from -3.7 kcal/mol to -4.3 kcal/mol. Notably, the entry with a binding affinity of -4.3 kcal/mol indicates the strongest interaction between PPAR gamma and linoleic acid, suggesting a high affinity for the receptor. Additionally, examining the RMSD values provides valuable information about the quality of the docking poses. The RMSD values measured with respect to both the unbound (rmsd/ub) and bound (rmsd/lb) states of the ligand indicate the extent of deviation from the optimal binding conformation (Meng *et al.*, 2012). Lower RMSD values, such as those observed in several entries, signify a closer alignment of the docked ligand with the receptor's binding site. These findings suggest that certain docking poses exhibit favorable binding orientations, potentially indicating regions of high complementarity between PPAR gamma and linoleic acid. Overall, these results shed light on the molecular interactions underlying the PPAR gamma ligand complex formation, offering valuable insights for understanding the ligand's pharmacological activity and therapeutic

potential. Further analysis and validation of these docking results could contribute to the development of novel PPAR gamma modulators with enhanced efficacy and specificity.

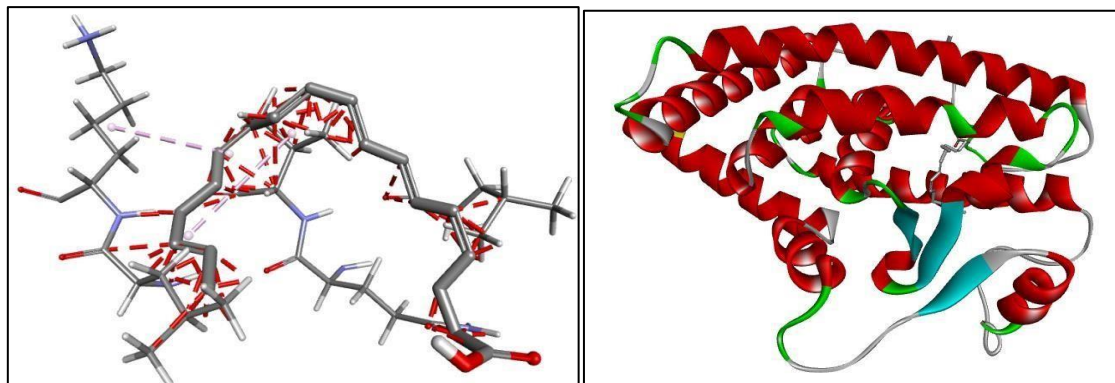


Figure 5.1: 3D structure of docked molecule

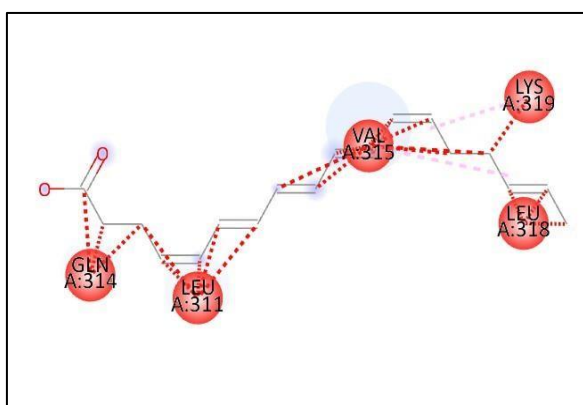


Figure 5.2: 2D structure of docked molecule

5.3 Cytotoxicity Assay

In order to determine the effects of linoleic acid on cytotoxicity, cells were incubated following 10, 20, 30, 40, 50, 60, 70 $\mu\text{g/mL}$ for 24 hours. The data obtained showed that there was a significant difference in cytotoxicity at 10, 20, 30, 40, 50, 60, 70 $\mu\text{g/mL}$ does compared to the control group. Compared to C2C12 (normal cell line) MDA-MB-231 (cancer cell line) showed higher percentage of cytotoxicity at various concentrations (Rose *et al.*, 1995). This shows that there is a higher sensitivity against linoleic acid for MDA-MB-231 compared to C2C12 cells. The half inhibition concentration (IC_{50}) values of linoleic acid in C2C12 cells and MDA-MB-231 cells were 50 μg and 41 μg

respectively. Given that MDA-MB-231 breast cancer cells had a lower IC₅₀ value than C2C12 cells, it appears that linoleic acid has a greater inhibitory effect on the growth or survival of breast cancer. The difference in IC₅₀ values suggests that linoleic acid may target breast cancer cells more successfully than normal cells. The observed discrepancy between malignant and normal cells may result from differences in cellular metabolism, genetic composition or signaling pathways.

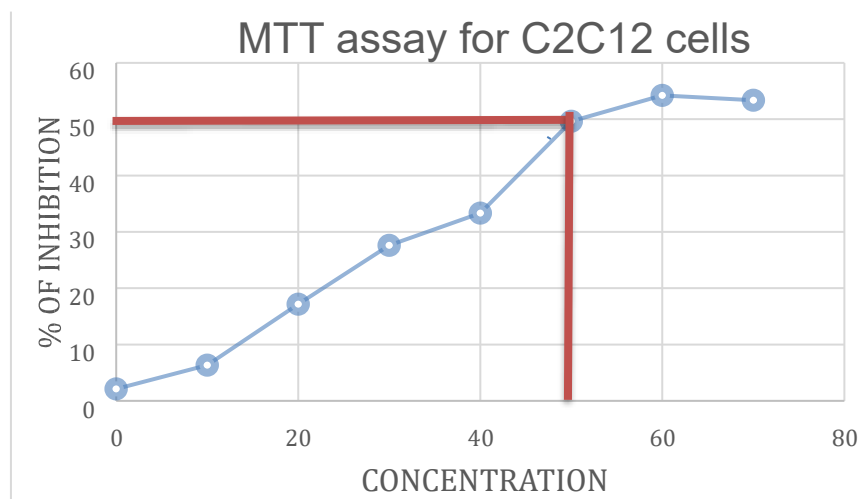


Figure 5.3: Assessment of Anti-cancer Activity of Linoleic Acid on C2C12 cell line

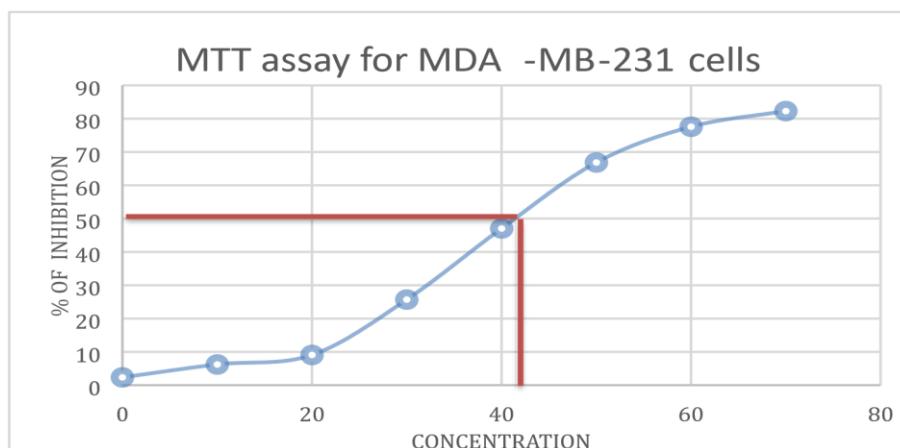


Figure 5.4: Assessment of Anti-cancer Activity of Linoleic Acid on MDA-MB231 cell line

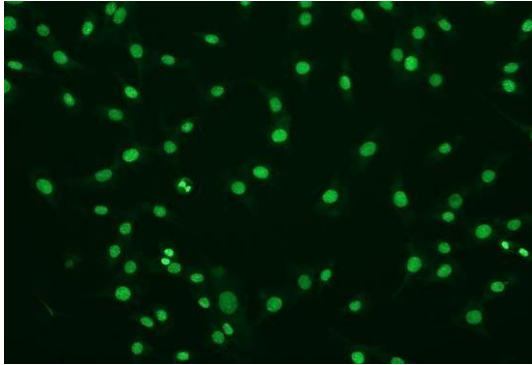
5.4 Acridine Orange/Ethidium Bromide (AO/EB) Fluorescent Staining

Upon observation, it was noted that in Control 1 and Treated 1 wells where only AO stain was added, there was a higher abundance of live cells compared to Treated 1 wells. This suggests that linoleic acid treatment may have an adverse effect on cell viability, leading to a decrease in the number of live cells.

In Control 2 and Treated 2 wells where only EB stain was added, the staining revealed cellular features indicative of late apoptotic and necrotic cells. Interestingly, there was a higher proportion of late apoptotic and necrotic cells in Treated 2 wells compared to Control 2 wells. This observation implies that linoleic acid treatment may induce apoptosis and necrosis in cancer cell lines. In Control 3 and Treated 3 wells where a combination of both AO and EB stains was added, differential staining allowed for the visualization of distinct cellular features associated with live, apoptotic, and necrotic cells. Consistent with the findings from Control 1 and Treated 1 wells, Control 3 wells exhibited a higher prevalence of live cells compared to Treated 3 wells. Additionally, the staining patterns in Treated 3 wells suggested an increased presence of late apoptotic and necrotic cells, further supporting the notion of linoleic acid-induced cell death (Galluzzi *et al.*, 2018). The observed differences in cell viability and apoptosis between control and treated groups indicate a potential cytotoxic effect of linoleic acid on cancer cell lines.

These findings underscore the importance of further investigating the mechanisms underlying linoleic acid-induced cytotoxicity and its potential therapeutic implications in cancer treatment. Further studies, including molecular analyses, are warranted to elucidate the pathways involved in linoleic acid-mediated cell death and to explore its therapeutic potential in cancer.

Control



Treated

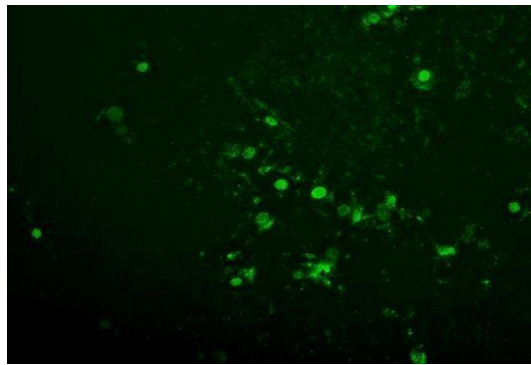


Figure 5.5: Control (Acridine Orange) **Figure 5.6:** Treated (Acridine Orange)

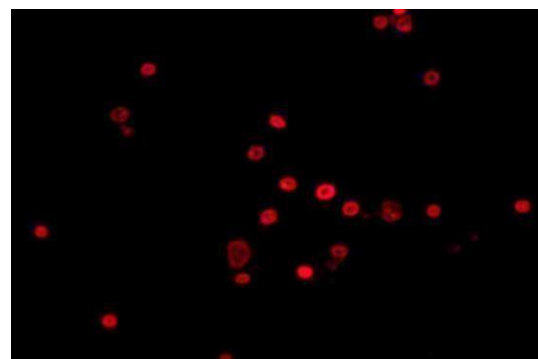
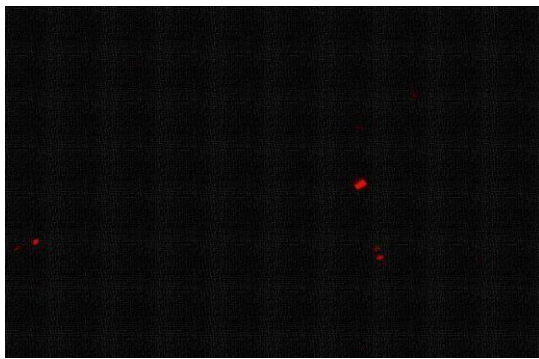


Figure 5.7: Control (Ethidium Bromide) **Figure 5.8:** Treated (Ethidium Bromide)

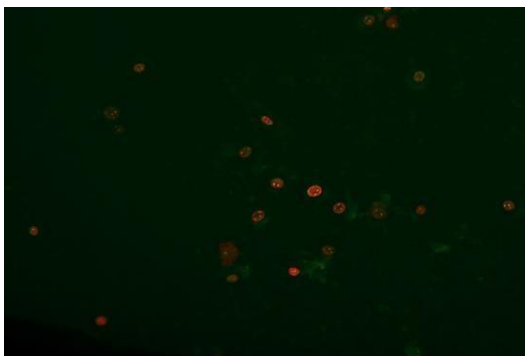


Figure 5.9: Control
(Ethidium Bromide + Acridine Orange)

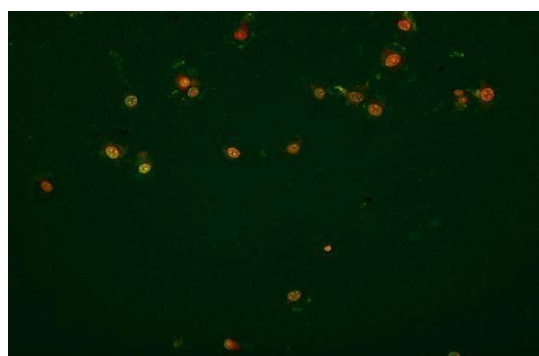


Figure 5.10: Treated
(Ethidium Bromide + Acridine Orange)

5.5 Total RNA isolation and cDNA Conversion

The process of isolating total RNA from MDA-MB-231 cells treated with linoleic acid involves several critical steps to ensure the integrity and purity of the RNA sample. Following treatment, cells were lysed, and total RNA was extracted using a suitable method, such as trizol or a commercial RNA isolation kit. The extracted RNA was then quantified using a spectrophotometer or fluorometric assay to assess its concentration and purity.

The quantified RNA was subsequently subjected to cDNA conversion using the iScript Reverse Transcription Kit under the specified conditions. This kit provides a convenient and efficient method for converting RNA into complementary DNA (cDNA) using reverse transcriptase enzyme and a mix of necessary components. The reaction conditions include a priming step at 25°C for 5 minutes, followed by reverse transcription at 46°C for 20 minutes to synthesize cDNA from the RNA template. Finally, the reaction was terminated by incubating at 95°C for 1 minute to inactivate the reverse transcriptase enzyme. The successful total RNA isolation and cDNA conversion from linoleic acid-treated MDA-MB-231 cells provide a foundation for further investigations into the molecular mechanisms underlying the effects of linoleic acid on gene expression in cancer cells.

Table 5.9: Components of cDNA conversion Kit

COMPONENT	VOLUME PER REACTION μ l
5X iScript Mix	4
iScript Reverse Transcriptase	1
RNA template	15
Total volume	20

Table 5.10: Protocol for cDNA conversion

Priming	5 min at 25°C
Reverse Transcription	20 min at 46°C
RT inactivation	1 min at 95°C
Optional step	hold at 4°C



Figure 5.11: Converted cDNA

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

Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) was done to check the inflammatory gene expression. GADPH gene (housekeeping gene) is used as control to compare it with other inflammatory gene. The cycle threshold was obtained and fold change was calculated using the below formulae

FORMULAE

$$\Delta C_t = C_t (\text{House-keeping gene}) - C_t (\text{gene of interest})$$

$$\Delta \Delta C_t = \Delta C_t - \text{Average } \Delta C_t$$

$$RQ = 2^{\Delta \Delta C_t}$$

Table 5.11: Results for qRT-PCR

Sample	Relative quantification
control	1
TNF- α	0.130894
IL-6	0.151878
IL-1 β	0.204883
NF-kb	0.191916

Linoleic acid treatment induces significant alterations in gene expression profiles, highlighting its potential as an agent with anticancer activity. Upon comparative analysis of relative quantification (RQ) values, which represent the fold change in expression levels of target genes compared to the housekeeping gene GAPDH, distinct downregulation patterns emerge in untreated cancer cells. Specifically, TNF- α , IL-6, IL1 β , and NF-kB exhibit notably lower RQ values relative to GAPDH in the control group. For instance, TNF- α displays an RQ value of 0.130894, IL-6 of 0.151878, IL-1 β of 0.2048831, and NF-kB of 0.1919164, signifying their suppressed expression levels compared to the reference gene (Schefe *et al.*, 2006). These findings suggest that linoleic acid treatment may modulate key pro-inflammatory and pro-survival signaling pathways, potentially contributing to its observed anticancer effects.

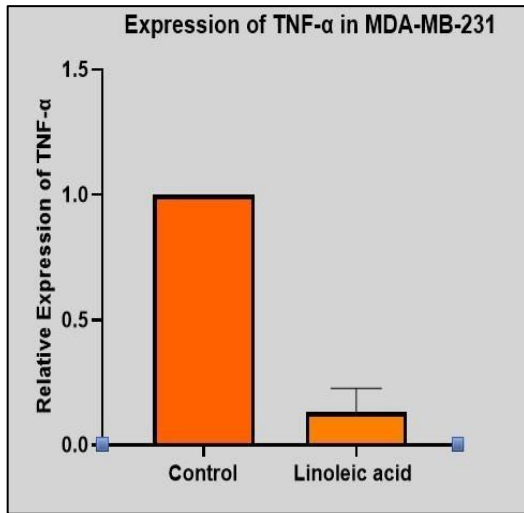


Figure 5.12: Expression of TNF- α

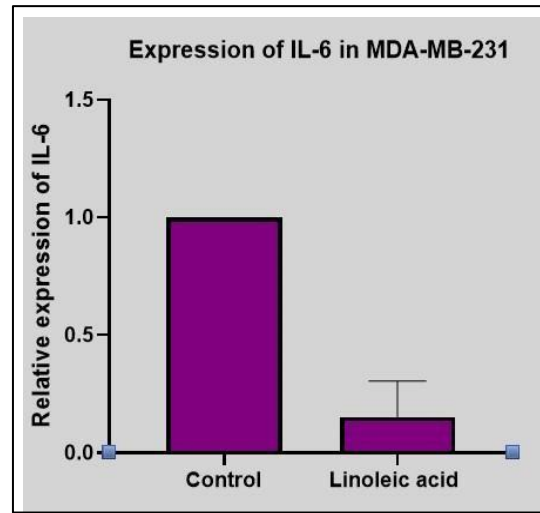


Figure 5.13: Expression of IL-6

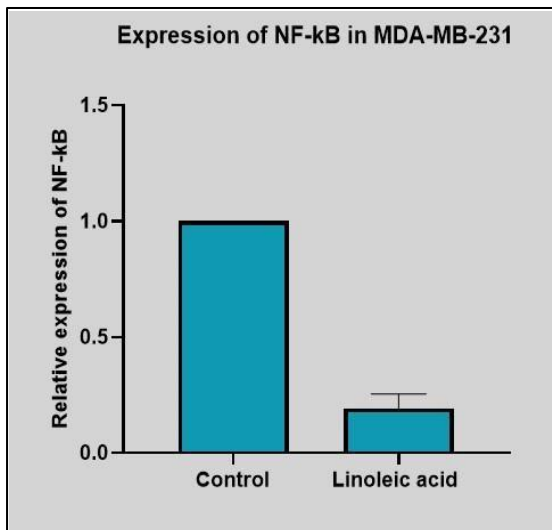


Figure 5.14: Expression of NF- κ b

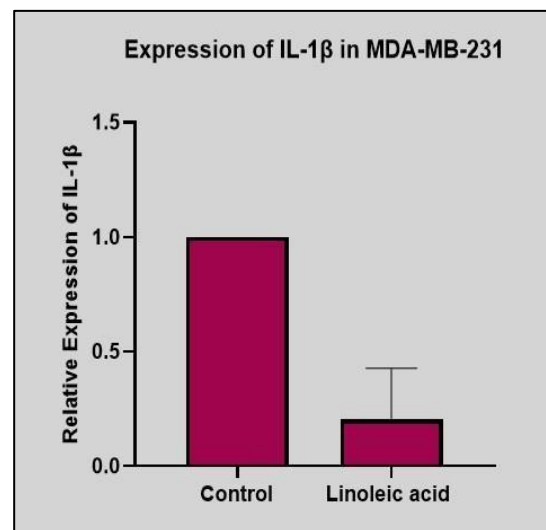


Figure 5.15: Expression of IL-1 β

5.7 Statistical analysis

This part covers the student outcome that indicates the ability to frame hypotheses, and use scientific judgement to draw conclusions.

5.7.1 Student t-test for cytotoxicity assay

Null hypothesis H_0 = There was no significant changes in % of inhibition within C2C12 normal cell line in different concentrations after 24hours

Alternative hypothesis H_a = There are significant changes in % of inhibition within C2C12 normal cell line in different concentrations after 24hours In this statistical analysis, student t-test was conducted to analyze the percentage of inhibition in C2C12 normal cell lines exposed to different concentrations of a substance over a 24-hour period. The choice of a t-test was appropriate for comparing means across different concentration groups. With a significance level (α) set at 0.05, aimed to determine whether there were significant differences in inhibition levels. The null hypothesis (H_0) posited no significant change in inhibition across concentrations, while the alternative hypothesis (H_a) suggested otherwise. By obtaining a p-value of 0.0028, which was less than the significance level, null hypothesis was rejected, indicating that there were indeed significant differences in inhibition levels among the various concentrations tested. This implies that exposure to different concentrations of the substance had a measurable effect on inhibiting cell growth or activity in the C2C12 cell line.

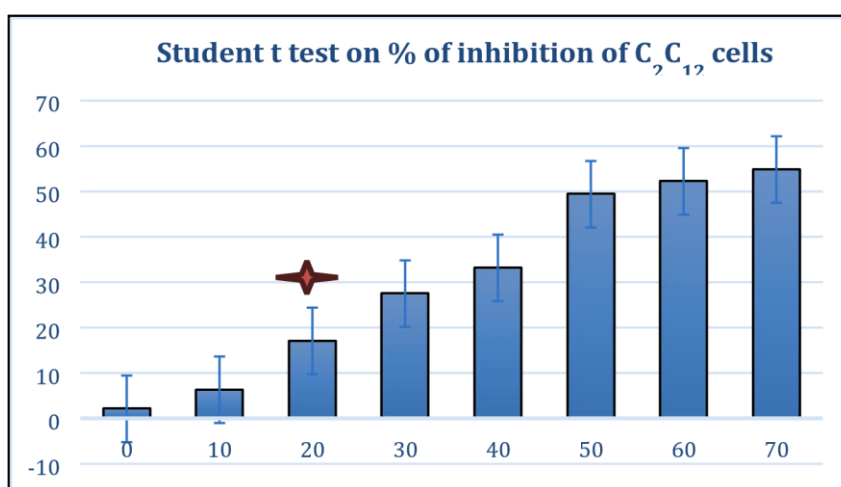


Figure 5.16: Student t test on % of inhibition of C2C12 cells

Null hypothesis H_0 = There was no significant changes in % of inhibition within MDAMB-231 cancer cell line in different concentrations after 24hours

Alternative hypothesis H_a = There are significant changes in % of inhibition within MDA-MB-231 cancer cell line in different concentrations after 24hours

In this statistical analysis, student t-test was conducted to analyze the percentage of inhibition MDA-MB-231 cancer cell in exposed to different concentrations of a substance over a 24-hour period. The choice of a t-test was appropriate for comparing means across different concentration groups. With a significance level (α) set at 0.05, aimed to determine whether there were significant differences in inhibition levels. The null hypothesis (H_0) posited no significant change in inhibition across concentrations, while the alternative hypothesis (H_a) suggested otherwise (Bland & Altman, 1995). By obtaining a p-value of 0.0003, which was less than the significance level, null hypothesis was rejected, indicating that there were indeed significant differences in inhibition levels among the various concentrations tested. This implies that exposure to different concentrations of the substance had a measurable effect on inhibiting cell growth or activity in the MDAMB-231 cancer cell.

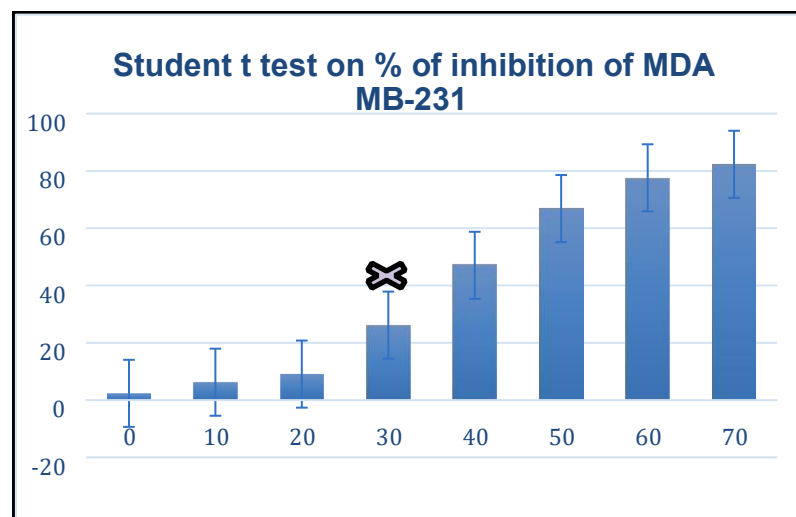


Figure 5.17: Student t test on % of inhibition of MDA-MB-231

Null hypothesis H_0 = There was no significant changes in % of inhibition of different concentration between C2C12 cell line and MDA-MB-231 cell line after 24hours

Alternative hypothesis H_a = There are significant changes in % of inhibition of the MDA-MB-231 cancer cell line in different concentrations after 24hours

In this statistical analysis, student's t-test between the groups was performed to compare the percentage of inhibition between two different cell lines, C2C12 and MDAMB231, across various concentrations after 24 hours. Your null hypothesis (H_0) stated that there would be no significant changes in the percentage of inhibition between these cell lines at different concentrations, while the alternative hypothesis (H_a) proposed that there would be significant changes (Ashby, 1991). A significance level of 0.05, indicates that acceptance 5% chance is standard for rejecting the null hypothesis. After conducting the t-test, p-value of 0.0005 was obtained. This p-value indicates that the probability of observing the results you obtained if the null hypothesis were true is very low. Therefore, based on your chosen significance level, you reject the null hypothesis. This implies that there are indeed significant differences in the percentage of inhibition between the two cell lines at different concentrations after 24 hours. This shows that the cellular response to the inhibitory agent varies significantly between the C2C12 and MDA-MB231 cell lines, indicating potential differences in their sensitivity or resistance to the treatment.

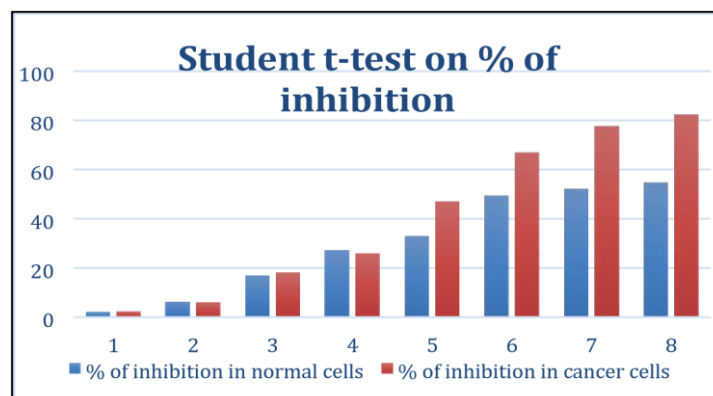


Figure 5.18: Student t-test on % of inhibition C2C12 cell line and MDA-MB-231

5.7.2 Chi-square test for AO/EB Staining

Chi-square analysis can be utilized in the context of AO/EB (acridine orange/ethidium bromide) staining to assess the viability of cells or organisms. AO/EB staining is commonly used in fluorescence microscopy to distinguish between live, apoptotic, and dead cells based on their nuclear morphology and membrane integrity. A $p\text{-value} < 0.0001$ obtained from an unpaired t-test indicates that there is a statistically significant difference between the control group and treated (linoleic acid) being compared this suggests that the likelihood of observing such a difference due to random chance, assuming the null hypothesis (no difference between groups) is true. Since the p-value is less than the conventional significance level of 0.05, we reject the null hypothesis. Therefore, the results of the unpaired t-test indicate a statistically significant difference ($p < 0.0001$) in staining between the control and treated groups. Hence, we reject the null hypothesis and accept the alternative hypothesis, suggesting that the treatment has a significant effect on the staining compared to the control group.

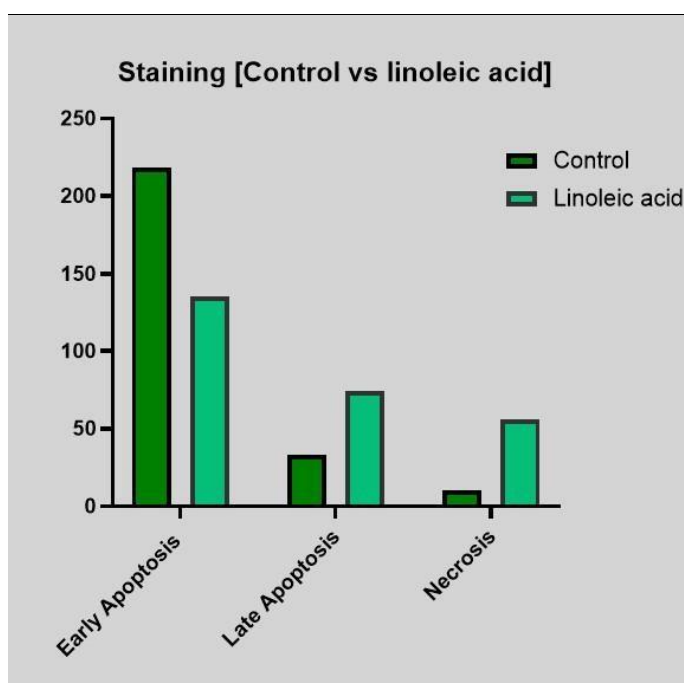


Figure 5.19: chi-square test for AO/EB staining

5.7.3 Unpaired t-test for Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)

The unpaired t-test is a statistical method used to compare the means of two independent groups. In this case, it was employed to analyze the quantitative reverse transcription polymerase chain reaction (qRT-PCR) results for the expression levels of inflammatory genes, namely IL-1 β , NF-kB, TNF- α , IL-6 (Livak & Schmittgen, 2001). The decision to use an unpaired t-test indicates that the samples being compared are not related or paired in any way. Each gene expression level was measured independently in distinct experimental conditions or samples, hence justifying the use of this statistical test. The obtained p-values for IL-1 β , NF-kB, IL-6, and TNF- α were 0.037, 0.005, 0.01, and 0.0059 respectively. These p-values represent the probability of observing the obtained data, or something more extreme, assuming the null hypothesis (no difference between groups) is true. Given the conventional significance level of 0.05, the null hypothesis is rejected for IL-1 β , NF-kB, IL-6, and TNF- α gene expressions. This rejection signifies that there are statistically significant differences in the expression levels of these inflammatory genes between the groups being compared. Therefore, the alternative hypothesis, which posits that there are indeed significant differences in gene expression between the groups, is accepted.

CHAPTER 6

SUMMARY AND CONCLUSION

The project titled "Targeting Breast Cancer Using Linoleic Acid and Its Effect on Inflammatory Gene Expression Studies" investigated the potential of linoleic acid as a therapeutic agent against breast cancer cells, focusing on its impact on cell viability, gene expression, and inflammatory pathways.

Firstly, the study explored the cytotoxic effects of linoleic acid on breast cancer cells (MDA-MB-231) compared to normal cells (C2C12). Results from cell viability assays revealed a concentration-dependent decrease in cell viability, with MDA-MB231 cells exhibiting higher sensitivity to linoleic acid-induced cytotoxicity compared to C2C12 cells. Moreover, the calculated IC₅₀ values indicated a stronger inhibitory effect of linoleic acid on breast cancer cells, suggesting its potential as a selective anticancer agent. Further investigations into the molecular mechanisms underlying linoleic acid's anticancer activity revealed alterations in the expression levels of inflammatory genes, including TNF alpha, IL-6, IL-1beta, and NF-kB. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis demonstrated significant downregulation of these genes in linoleic acid-treated breast cancer cells, indicative of its ability to modulate pro-inflammatory and pro-survival signaling pathways. Additionally, molecular docking studies provided insights into the interaction between linoleic acid and PPAR gamma, a key nuclear receptor involved in regulating inflammatory responses and cell proliferation. Docking results indicated favorable binding affinities, suggesting potential molecular mechanisms underlying linoleic acid's anti-inflammatory and anticancer effects.

Furthermore, AO/EB fluorescent staining revealed linoleic acid-induced alterations in cell morphology and viability, with increased apoptosis and necrosis observed in treated breast cancer cells compared to controls. Statistical analyses, including student t-tests and chi-square tests, confirmed

the significance of the observed findings, supporting the efficacy of linoleic acid in targeting breast cancer cells and modulating inflammatory pathways.

In conclusion, this study provides comprehensive evidence supporting the potential of linoleic acid as a promising therapeutic agent for breast cancer treatment. Its ability to induce cytotoxicity, modulate inflammatory gene expression, and interact with key molecular targets highlights its multifaceted anticancer properties. Further research into the underlying mechanisms and in vivo efficacy of linoleic acid is warranted to validate its clinical utility and optimize its therapeutic potential in breast cancer management.

Research can be explored to further elucidate the therapeutic potential of linoleic acid in breast cancer treatment and expand our understanding of its mechanisms of action. Firstly, conducting in vivo studies using animal models of breast cancer can provide valuable insights into the efficacy and safety of linoleic acid as a therapeutic agent. By evaluating its effects on tumor growth, metastasis, and overall survival, these studies can validate the preclinical findings and inform clinical trial design. Moreover, exploring the synergistic effects of linoleic acid with other anticancer agents or treatment modalities holds promise for enhancing its therapeutic efficacy. Combination therapies involving linoleic acid and conventional chemotherapeutic agents, targeted therapies, or immunotherapies could potentially improve treatment outcomes and overcome resistance mechanisms in breast cancer patients.

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ANNEXURE



Plagiarism Checker X - Report

Date: Mar 30, 2024
Matches: 5 / 119 words
Sources: 1

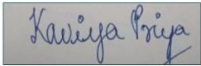
Originality Assessment
4%

Remarks: Low similarity detected, consider making necessary changes if needed.
Verify Report:
Scan this QR Code



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Your GATE 2024 Result [BT]

Name	KAVIYA PRIYA K	
Registration Number	BT24S57401227	
Gender	Female	
Parent's/Guardian's name	KANDEEBAN V	
Date of Birth (YYYY-MM-DD)	2003-02-11	
2003-02-11		
Examination Paper	Biotechnology (BT)	
Marks out of 100 [#]	19.33	
Qualifying Marks ^{##}	38.9 35 25.9 General OBC-NCL/EWS SC/ST/PwD	Signature

[#]Normalized marks in case of multisection papers (CE and CS).

^{##}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

CLOSE

REGISTER NUMBER	11553347
GENDER	Female
NATIVITY	Tamil Nadu
DATE OF BIRTH	11/02/2003
COMMUNITY	BC
NATIONALITY	Indian
ENTRANCE TEST	M.E/M.Tech/M.Arch/M.Plan
SUBJECT CHOICE	10-Bio-Technology



Kaviya Priya

EXAM	ACTUAL SCORE	PERCENTILE SCORE	NUMBER OF CANDIDATES APPEARED
MCA	---	---	---
MBA	---	---	---
ME(ON SUBJECT CHOICE)	23.5	84.911	338
ME(OVERALL)		83.111	4648

- Percentile Score will be considered for Merit list.
- Score card will not be sent by post or Email.
- For M.E/M.Tech, overall percentile score will be considered for Merit List.
- For M.E/M.Tech overall percentile score is calculated after normalization.
- Percentile Score is the percentage of number of candidates scored less than your score.
- Score card will be released on **April 03, 2024** and that will be available till **May 03, 2024**.
- TANCET (MBA / MCA) candidates have to apply separately for admission. The advertisement will be released by the respective admitting authorities.
- **CEETA-PG (M.E/M.Tech/M.Arch/M.Plan) candidates have to exercise the choice of college & branch in the web portal after notifications from Anna University.**