

**ANTI-CANCER EFFECT OF CYCLODIPEPTIDE ON HUMAN BREAST CANCER
CELL LINE THROUGH INVOLVEMENT OF NRF2 AND MITOCHONDRIAL
APOPTOSIS SIGNALING**

PROJECT WORK

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

By

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DEPARTMENT OF BIOTECHNOLOGY

SATHYABAMA

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **SOWMYA RAO P (41770041)** who carried out the project entitled "**ANTI-CANCER EFFECT OF CYCLODIPEPTIDE ON HUMAN BREAST CANCER CELL LINE THROUGH INVOLVEMENT OF NRF2 AND MITOCHONDRIAL APOPTOSIS SIGNALING**" under my supervision from January 2024 to April 2024.

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ABSTRACT

This present study presents a comprehensive investigation into the anticancer effects of cyclo(-Pro)(-Tyr), a cyclical dipeptide, on human breast cancer cell lines, with a focus on its molecular mechanisms and therapeutic potential. This study meticulously evaluates the dipeptide's ability to modulate key signaling pathways, particularly NRF2 and the mitochondrial apoptotic pathways, which play crucial roles in the cellular stress response and apoptosis regulation. The findings indicate that cyclo(-Pro)(-Tyr) significantly inhibits proliferation and induces apoptosis in treated breast cancer cells, suggesting its potential utility as a therapeutic agent against breast cancer. The research extends to examining the effects of cyclo(-Pro-Tyr) on the oxidative stress response, enhancing the understanding of its role in maintaining cellular redox homeostasis through NRF2 pathway modulation. This dipeptide also impacts mitochondrial function, triggering apoptotic processes critical for cancer cell elimination. By delving into the cellular and molecular responses of breast cancer cells to cyclo(-Pro-Tyr) treatment, the thesis highlights the compound's dual functionality in inhibiting cancer cell growth and promoting apoptotic cell death. This comprehensive approach not only validates the anticancer properties of cyclo(-Pro-Tyr) but also underscores its potential to be developed into a targeted therapy that could offer specificity in cancer treatment, reducing the side effects associated with conventional chemotherapy. The findings contribute significant new insights to the field of oncology, particularly in the development of peptide-based therapies, and pave the way for future studies to explore its efficacy in clinical settings.

Keywords: Cyclo(-Pro-Tyr), Breast cancer, Apoptosis, NRF2 Signalling, Dipeptide, Proliferation, Metastasis.

TABLE OF CONTENTS

Sno.	CONTENTS	PAGE No
	Abstract	i
	List of abbreviations	v
	List of figures	viii
	List of tables	ix
1	INTRODUCTION	1
1.1	Introduction to cancer	1
1.1.2	Causes of cancer	2
1.1.2.1	Sunlight	2
1.1.2.2	Tobacco	3
1.1.2.3	Alcohol	3
1.1.2.4	Obesity	4
1.1.3	Common symptoms of cancer	4
1.1.4	Types of cancer and cancer statistics	4
1.2	Treatment of cancer	5
1.2.1	Surgery	5
1.2.2	Chemotherapy	6
1.2.3	Radiation therapy	6
1.2.4	Immunotherapy	6
1.2.5	Targeted therapy	6
1.3	Cancer statistics	7
1.4	Breast cancer	8
1.4.1	Statistics	9
1.4.2	Types of breast cancer	9
1.4.2.1	Based on ability to spread	9
1.4.2.2	Based on molecular make-up	10

2 LITERATURE SURVEY	11
2.1 Metastatic breast cancer	11
2.2 Potential targeted treatment for breast cancer	11
2.3 Cyclo(-Pro-Tyr)	13
2.4 Drug-induced apoptosis of cancer cells	14
2.5 Role of NRF2 in breast cancer cells	16
3 AIM AND OBJECTIVES	18
3.1 Aim	18
3.2 Objectives	18
4 MATERIALS AND METHODS	19
4.1 Chemical reagents	19
4.2 Invitro cell line studies	19
4.2.1 Cell line maintenance	20
4.2.2 Experimental Design	21
4.2.3 Cell viability and cytotoxicity assay	21
4.2.4 Necrosis assay by PI staining	22
4.2.5 Apoptosis Assay	23
4.2.6 ROS staining	23
4.2.7 RNA Isolation	24
4.2.8 cDNA conversion	25
4.2.9 Primer designing	26
4.2.10 RT-PCR analysis	27
4.2.11 DNA fragmentation analysis	2
4.2.12 SDS-PAGE analysis	29
5 RESULTS AND DISCUSSION	31
5.1 Cell viability and cytotoxicity assay	31
5.2 Cell imaging	32
5.3 PI staining	33
5.4 AO/EB apoptosis staining	34
5.5 DCFH-DA staining	35

5.6 RT-PCR analysis	36
5.7 DNA fragmentation analysis	38
5.8 SDS-PAGE analysis	39
5.9 Discussion	40
6 SUMMARY AND CONCLUSION	42
REFERENCES	43

LIST OF ABBREVIATIONS

ACTB	Actin beta/ β -Actin
AKT	Protein kinase B
AO	Acridine Orange
BAX	Bcl-2-associated protein x
BCC	Breast cancer cells
BCL2	B-cell leukaemia/lymphoma 2 protein
BP	Base pair
CASP3	Caspase-3
CBB	Coomassie brilliant blue
cDNA	Complementary DNA
CPT	Cyclo(-Pro-Tyr)
DCHF-DA	Dichloro-dihydro-fluorescein diacetate
DKP	Cyclic dipeptide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DP	Dipeptide
ER	Estrogen receptor
EtBr/EB	Ethidium Bromide
FBS	Fetal bovine serum
GLOBOCAN	Global Cancer Observatory

GSH	Glutathione
H ₂ O ₂	Hydrogen peroxide
HER	Human epidermal growth factor receptor 2
IC ₅₀	Half-maximal inhibitory concentration
LDH	Lactate dehydrogenase
mL	Milli litre
mRNA	Messenger Ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCCS	National Centre for Cell Science
NRF2	Nuclear factor erythroid 2-related factor 2
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinases
PR	Progesterone receptor
QoL	Quality of life
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RQ	Relative quantification
RT PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation

SDS	Sodium dodecyl Sulphate
TNBC	Triple Negative Breast Cancer
TNF	Tumour necrosis factor
TXN	Thioredoxin
UV	Ultra-Violet
ZnO	Zinc Oxide
ZnONP	ZnO nanoparticles

LIST OF FIGURES

Sno.	FIGURES	PAGE No
1.1	Different UV rays produced by the sun	2
1.2	Cancers associated with drinking alcohol	3
1.3	Statistics on case incidence of different cancers in 2023	8
1.4	The process of metastasis	10
4.1	Experimental design	21
4.2	Cell viability assay	21
4.3	RNA isolation	25
4.4	cDNA conversion	25
4.5	Agarose Gel electrophoresis	28
4.6	Schematic diagram of SDS-PAGE	30
5.1	Cell viability and cytotoxicity assay	31
5.2	Phase contrast images of MDA-MB-231 cells	32
5.3	PI staining of MDA-MB-231 cells	33
5.4	AO/EB staining of MDA-MB-231 cells	34
5.5	DCFH-DA staining of MDA-MB-231 cells	35
5.6	RT-PCR gene expression analysis of different gene markers	36,37
5.7	Agarose gel visualisation of DNA bands	38
5.8	SDS PAGE gel staining images	39

LIST OF TABLES

Sno.	TABLES	PAGE No.
4.1	List of cell lines used	20
4.2	Gene expression primers by qPCR analysis	27

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO CANCER

In our body, there are around 30 trillion cells. Each cell divides, grows, and performs specific functions to keep our body alive and ultimately, die. But not every cell is able to carry out this cycle. Some cells die before performing their duty and some cells, on the other hand, simply do not die like a cancer cell. Cancer is a set of diseases that progress by repeated mutations in bodily cells causing them to malfunction. These malfunctions may include rapid uncontrolled cell proliferation, modified metabolism to support the rapid proliferation, changes in expression levels of different regulatory, and altered cell life span (Cree IA, 2011). Generally, when a cell is created, it dies after a certain period of time. However, a mutated cancer cell has a prolonged life span. This characteristic along with rapid proliferation results in an increment of mutated cancerous cells in our body. These cells often accumulate in specific spaces in our body, forming something called a tumor. A tumor is a mass of cancer cells that lodges at various regions in our body. Many times, the tumor disturbs the natural functions of our body. It can cause hindrance by blocking pathways physically (Chauhan VP *et al.*, 2013) or even by hijacking the body's homeostasis by disturbing the endocrinal and neural systems (Slominski RM *et al.*, 2023).

Now cancer is one of the deadliest diseases in humans, second only to cardiovascular disease. In 2020 alone, around 20 million cancers were diagnosed and over 10 million cancer-related deaths were reported, according to Sung H *et al.*, 2021. The cause of this deadly disease has been researched over decades and it is found that many factors bring about mutations forming cancer; namely, sunlight, tobacco, alcohol, hormones, red meat, obesity, viruses, etc. (Blackadar CB, 2016)

1.1.2 Causes of cancer

1.1.2.1 UV Exposure

The sun produces ultraviolet (UV) rays relentlessly. Our Earth's ozone layer usually deflects these UV rays. But as global warming increases, our ozone layer is getting depleted, resulting in harsher UV rays on the Earth's surface where we are. The majority of the studies show that regular exposure to UV radiation on the skin results in skin cancer or melanoma. Experiencing severe sunburns before the age of 25 has been linked to elevated risks of developing all types of skin cancers over the years (Savoye I *et al.*, 2017). This association underscores the importance of early sun protection practices in mitigating the long-term impact of harmful ultraviolet (UV) radiation exposure.

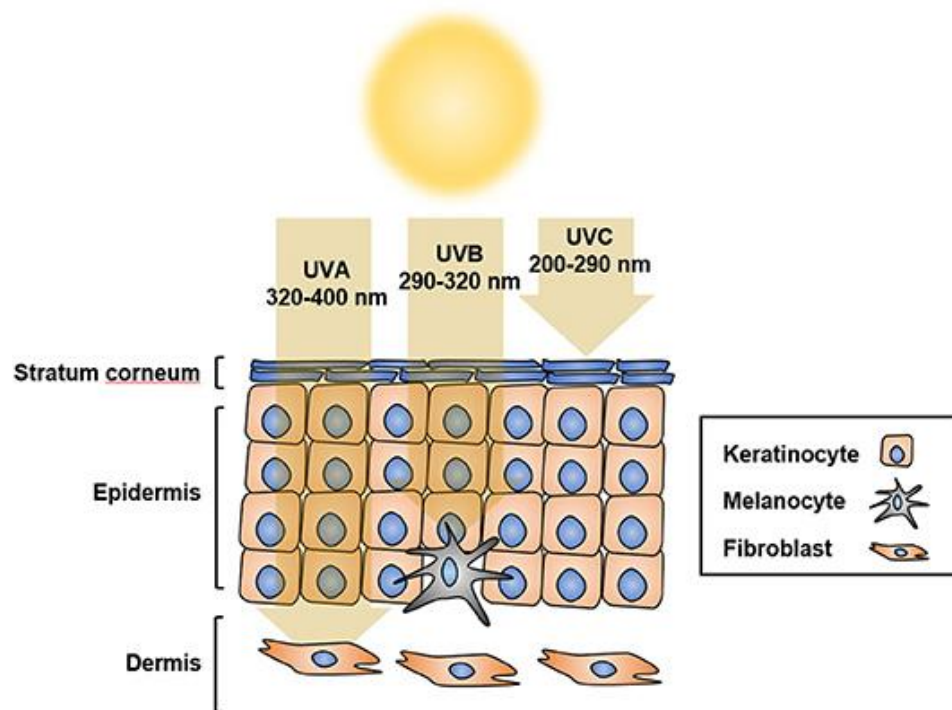


Fig 1.1 Different UV rays produced by the sun. The sun produces three different types of UV rays: ultraviolet A (UVA), ultraviolet B (UVB), and ultraviolet C (UVC). UVA and UVB rays pose a greater risk of sun damage (Sun X *et al.*, 2020).

1.1.2.2 Tobacco

Along with being the most preventable, Smoking tobacco is one of the leading causes of many different cancers. The carcinogens in the smoke come in contact with many different organs in our body from the oral cavity, esophagus to the lungs and bronchus. The risk of various cancers like lung, cervical, pancreas, bladder, kidney, and stomach cancers, seems to increase by 50 to 200 times in frequent smokers than in normal human beings (Scherübl H *et al.*, 2023).

1.1.2.3 Alcohol

Intensive alcohol consumption, defined as consuming four or more drinks per day, is notably linked to a heightened risk across various cancers. Specifically, this behavior increases the risk approximately fivefold for oral and pharyngeal cancer, as well as esophageal squamous cell carcinoma. Additionally, it raises the risk by 2.5 times for laryngeal cancer, by 50% for colorectal and breast cancers, and by 30% for pancreatic cancer. These findings underscore the significant impact of heavy alcohol intake on the development of multiple cancer types, emphasizing the importance of moderation in alcohol consumption for overall health and cancer prevention. The consumption of alcohol, primarily as ethanol, is recognized to induce cancer through several mechanistic pathways, although some remain incompletely elucidated.

(Pelucchi C *et al.*, 2011)

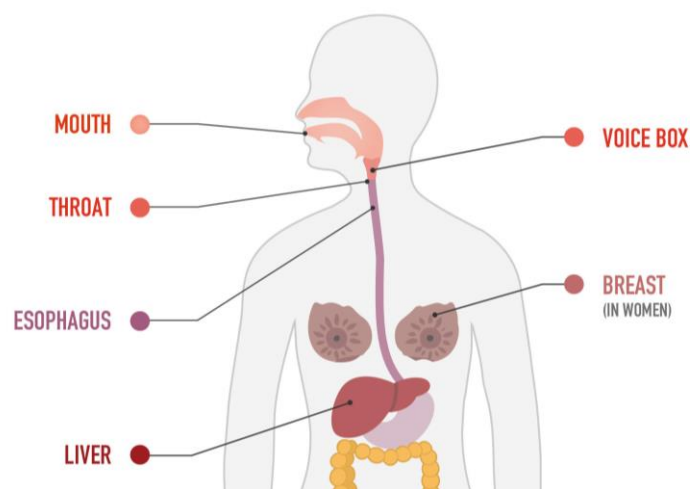


Fig 1.2 Cancers associated with drinking alcohol (National cancer institute)

1.1.2.4 Obesity

Obesity, or an excess of body fat, poses a significant global health concern, being linked not only to diabetes mellitus and cardiovascular disease but also serving as a prominent risk factor for the onset and deaths associated with a subset of cancers. Obesity is a risk factor for several major cancers, including post-menopausal breast, colorectal, endometrial, kidney, esophageal, pancreatic, liver, and gallbladder cancer. Too much body fat alone increases the risk of cancer-specific death by 17%. About 4–8% of all cancers are attributed to obesity. (Pati S *et al.*, 2023)

1.1.3 Common symptoms of cancer

Cancer patients may experience sudden changes in weight, reduced appetite, fatigue, depression, anxiety, reduced quality of life (QoL), and sleep problems. As the disease progresses to later stages, it can even end with death. Many cancers are mostly treatable during early stages but become harder and harder to treat as they progress. Cancer usually does not produce noticeable symptoms during its early stages. So by the time symptoms become perceivable the disease would have developed to its later stages. Therefore, early diagnosis is important but incredibly infrequent (Misiąg W *et al.*, 2022).

1.1.4 Types of cancer and cancer statistics

Based on the specific type of cell that turns cancerous, it is divided into 4 main types: Carcinoma, sarcoma, lymphoma, leukemia (Cooper GM, 2000). Carcinoma is the most common type of cancer diagnosed each year. Cancer from the epithelial cells that produce fluids or mucus is classified into the carcinoma subtype. This includes breast, kidney, uterus, skin, stomach, and intestinal cancers. All these organs have epithelial cells lining them and are classified as carcinoma (Hedberg ML *et al.*, 2022). Cancer formed in bones and soft tissues like muscle, lymph vessels, fat and blood tissues are called sarcoma. Osteosarcoma (bone cancer), liposarcoma (fat cell cancer), leiomyosarcoma (smooth muscle cell cancer) are some examples of sarcoma

(Banaszek N *et al.*, 2023). Our body has several defense mechanisms in our immune system. One such important defense mechanism is lymphocytes or white blood cells. These include B cells and T cells. Cancer in these cells is classified as lymphoma. Lymphoma entails the accumulation of abnormal lymphocytes in lymph nodes, lymph vessels, and various organs throughout the body. In our body, all our blood cells are formed in a tissue called the bone marrow. Cancer in these cells is classified as leukemia. In this type of cancer, there is a build-up of defective white blood cells in blood and bone marrow, blocking out normal blood cells. Cancer can be classified based on the origin of the organ as well. They are named after the organ too. For example, stomach cancer, lung cancer, breast cancer, etc.

1.2 TREATMENT OF CANCER

With advancements in technology, cancer treatments have evolved significantly, driven by an enhanced understanding of the underlying biological processes. Various treatment methods, including surgery, chemotherapy, radiation therapy, immunotherapy, and hormone therapy, have been practiced over the years. Emerging innovations, such as targeted therapy, continue to reshape contemporary cancer care, with ongoing efforts aimed at improving treatment effectiveness and precision.

1.2.1 Surgery

Surgery remains a primary treatment modality for non-hematological cancers, offering potential for curative resection. Surgeons aim to remove cancerous tissues, providing either complete or partial cure depending on factors like cancer type and patient health. Procedures vary based on cancer localization and size, with examples including mastectomy for breast cancer and prostatectomy for prostate cancer. However, complete removal may not be feasible if cancer has metastasized, underscoring the importance of evaluating residual cancer through biopsy (Benjamin DJ, 2014).

1.2.2 Chemotherapy

Chemotherapy employs drugs, known as anticancer drugs, to hinder tumor growth and destroy cancer cells. While effective, chemotherapy can induce severe side effects due to its impact on healthy cells. Treatment duration and drug combinations are tailored to individual needs, with combination chemotherapy being a common approach (Pérez-Herrero E *et al.*, 2015).

1.2.3 Radiation Therapy

Radiation therapy utilizes high doses of ionizing radiation to kill cancer cells and shrink tumors. It can be administered externally or internally and is often used in conjunction with surgery. However, radiation may also damage nearby healthy tissues, leading to side effects (Baskar R *et al.*, 2015).

1.2.4 Immunotherapy

Immunotherapy harnesses the body's immune system to combat cancer, stimulating its natural defense mechanisms. Techniques include monoclonal antibodies that target specific proteins on cancer cells, enhancing immune recognition and response. Immunotherapy generally boasts fewer side effects compared to traditional treatments (Riley RS *et al.*, 2019).

1.2.5 Targeted Therapy

In cancer treatment, targeted therapy specifically addresses the mechanisms that enable cancer cells to grow, divide, and spread. Unlike conventional chemotherapy, which affects both healthy and cancerous cells, targeted therapy focuses on inhibiting particular proteins or pathways essential for cancer cell survival and proliferation. These targeted agents are designed to disrupt the activity of deregulated proteins within cancer cells. Small molecule drugs, commonly used in targeted therapy, act as inhibitors targeting enzymatic domains associated with mutated, overexpressed, or critical proteins involved in cancer progression. By

selectively targeting cancer cells while minimizing harm to healthy tissues, targeted therapy offers the potential for more precise and effective treatment approaches in oncology (Lee YT *et al.*, 2019). These diverse treatment modalities underscore the multifaceted approach to cancer care, emphasizing tailored strategies to optimize patient outcomes and quality of life.

1.3 CANCER STATISTICS

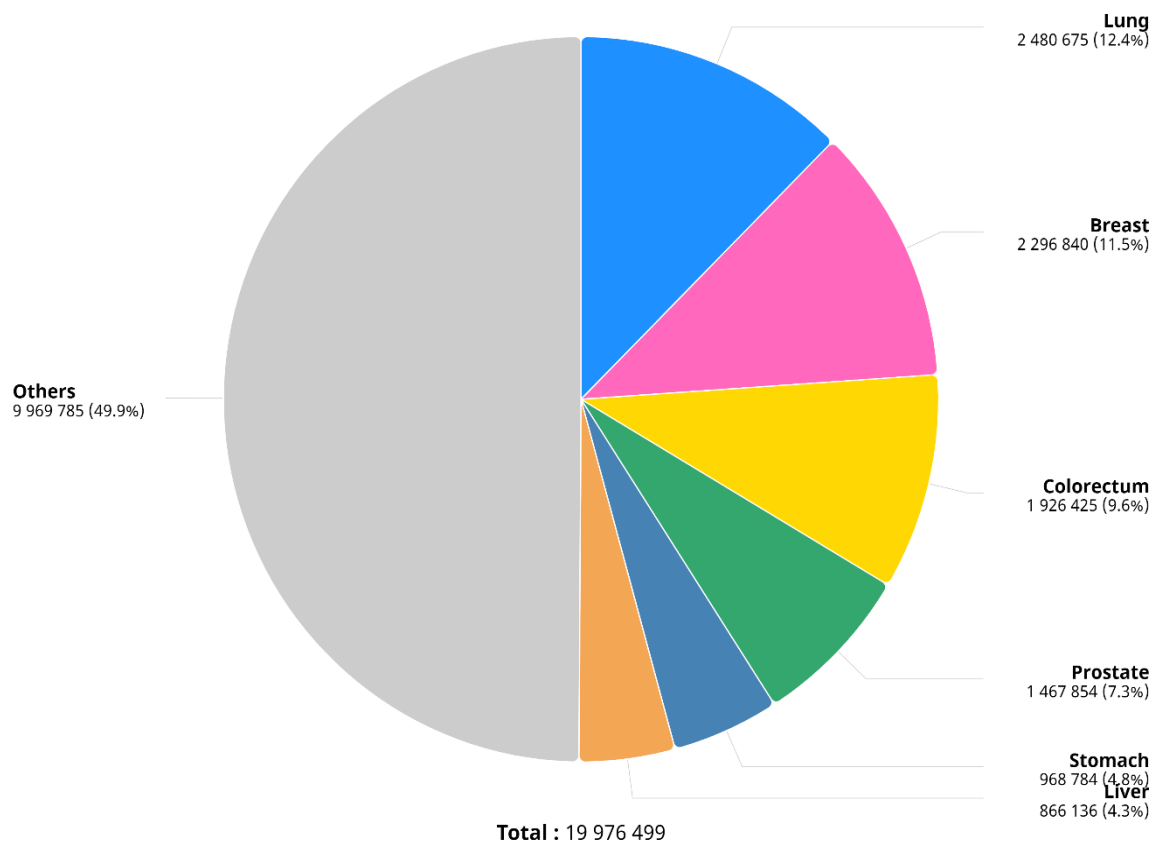


Fig 1.3 Statistics on case incidence of different cancers in 2023 (Globocan 2023)

According to the latest data from the Global Cancer Observatory (GLOBOCAN) 2022, there are 14 lakhs new diagnoses of cancer in India alone. Globally, over 1.9 crores new cases were observed. These numbers highlight the scale of the challenge posed by cancer worldwide, affecting individuals of all ages and demographics.

Regionally, the incidence and mortality rates vary, with higher rates typically observed in developed countries. Some of the most common types of cancer include lung, breast, colorectal, prostate, and stomach cancer. The total number of cancer-related deaths in 2022 is 97 lakhs. These statistics underscore the importance of continued efforts in cancer prevention, early detection, and treatment to reduce the global impact of this disease. In particular, breast cancer is the leading cause of cancer-related death in women. Breast cancer has surpassed lung cancer in number of cases of diagnosis as of 2020 (Sung H *et al.*, 2021).

1.4 BREAST CANCER

Breast cancer ranks as the most prevalent cancer among women, constituting over 10% of new cancer diagnoses annually. It stands as the second leading cause of cancer-related deaths in women globally. Often, breast cancer progresses without overt symptoms, with many cases detected during routine screenings. However, some individuals may notice telltale signs such as breast lumps, changes in breast size or shape, or nipple discharge. The disease arises from DNA damage and genetic mutations, which can be influenced by estrogen exposure. In certain cases, individuals may inherit DNA defects or predisposing genes heightening the risk of breast cancer. Notably, a family history of breast or ovarian cancer elevates susceptibility to the disease. In a healthy individual, the immune system identifies and eliminates cells with abnormal DNA or growth. However, this surveillance mechanism falters in breast cancer, enabling tumor growth and metastasis. (Alkabban FM *et al.*, 2022).

1.4.1 Statistics

Overall, breast cancer incidence rates increased by 0.79% annually over the study period of 2000 to 2019. Incidence increased gradually between 2000 and 2016 and then dramatically from 2016 onwards (Xu S *et al.*, 2024). Globally, in 2020, there were an estimated 23 lakhs new cases of breast cancer and approximately 6.8 lakhs

deaths from the disease. Also, in 2022, the new cases incidence has gone down to 22 lakhs and the deaths have decreased to 6.6 lakhs (Ferlay J *et al.*, 2024).

1.4.2 Types of breast cancer

1.4.2.1 Based on ability to spread

While a majority of breast cancer types remain benign, confined to their original site, approximately 10% to 15% of women diagnosed with early-stage cancer progress to malignant breast cancer. This transition occurs through metastasis, wherein cancer cells proliferate and infiltrate distant regions of the body, including the bones, lungs, liver, and brain, posing significant health challenges and complexities in treatment.

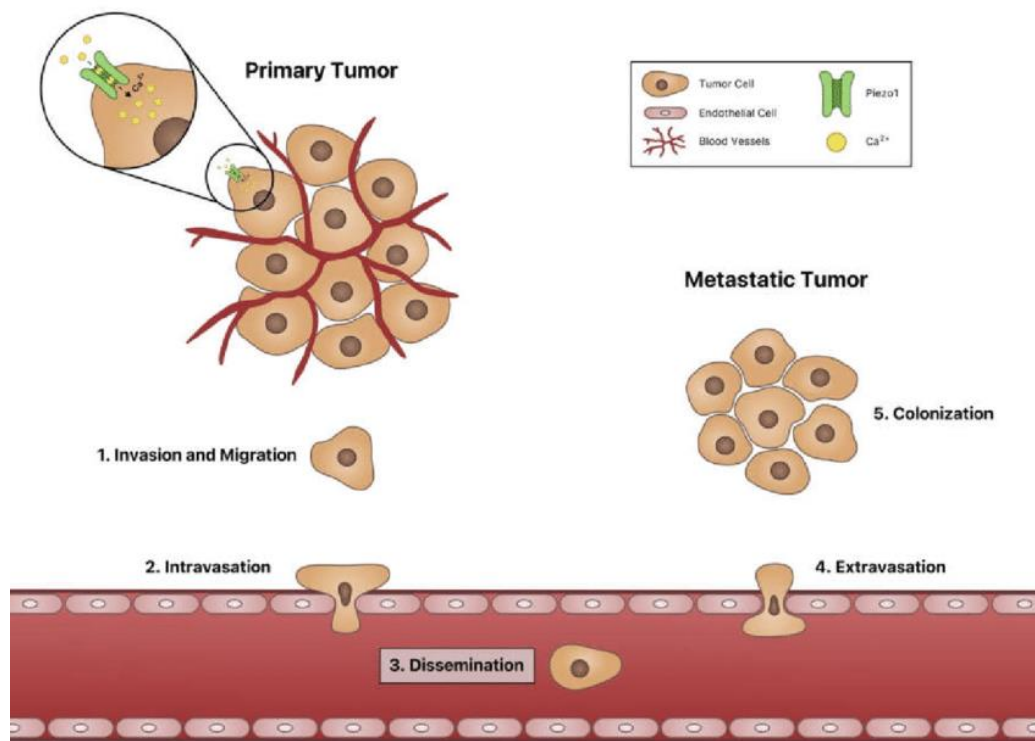


Fig 1.4 The process of metastasis is divided into 5 steps, namely:
i) Invasion and migration, ii) Intravasation, iii) Dissemination,
iv) Extravasation, v) Colonization (Dombroski JA *et al.*, 2021).

1.4.2.2 Based on molecular make-up

Metastatic breast cancer cells are classified molecularly according to the expression levels of hormone receptors. This includes estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and triple-negative breast cancer (TNBC). This immuno-histological classification yields subtypes, namely: Luminal A (PR-positive and HER2-negative tumors), Luminal B (ER and HER2-positive tumors), Basal-like (ER and HER2-negative), and HER2-enriched (Yersal O *et al.*, 2014).

CHAPTER 2

LITERATURE SURVEY

2.1 METASTATIC BREAST CANCER

In the review by (Łukasiewicz S *et al.*, 2021) they aimed to summarize and update the current knowledge about breast cancer, with a particular emphasis on its epidemiology, risk factors, classification, prognostic biomarkers, and available treatment strategies. Given the significant increase in both the morbidity and mortality rates of breast cancer over recent decades, there exists an urgent need to identify the most effective prevention measures, especially considering the potential impact of modifiable risk factors in reducing breast cancer incidence. Presently, mammography and sonography are the most commonly utilized screening tests, facilitating early detection of breast cancer. The ongoing pursuit of prognostic biomarkers and targets for potential biological therapies has notably contributed to the improvement of management and clinical outcomes for breast cancer patients.

In an article by Lian Y, *et al.*, 2020, the authors convey that patients diagnosed with metastatic breast cancer often do not receive timely targeted therapy due to delays in detection compared to the onset of metastasis. Thus, intervening during the early stages of distant metastasis, such as colonization and growth, could significantly enhance patient survival rates. A comprehensive understanding of metastatic heterogeneity mechanisms is vital for devising effective strategies to combat metastasis. Nonetheless, further research is essential to validate the identified genes and molecular mechanisms for future clinical applications.

2.2 POTENTIAL TARGETED TREATMENT FOR BREAST CANCER

The EPHOS-B Trial conducted by Bundred N *et al.*, 2022 successfully achieved one of its primary objectives by demonstrating that 11 days of anti-HER2 therapy on breast cancer patients, administered between diagnosis and surgery without

chemotherapy, led to a reduction in proliferation. This effect was observed across all active treatment groups, with particularly noteworthy results seen in the dual-agent combination group, where Ki67, a cellular marker for proliferation, was decrease of over 30% was observed in 74% of cases. Additionally, some tumors exhibited significant size reduction, rendering them too small for analysis at the time of surgery. Exploratory analysis further revealed that dual blockade with lapatinib and trastuzumab resulted in 6% of cases showing no residual invasive disease in the breast or nodes. with an additional 20% of cases exhibiting only minimal residual disease.

In a research conducted by Jamalzadeh L *et al.*, 2017, The MTT assay results reveal that artesunate significantly inhibits the growth of MCF-7 cells in a dose- and time-dependent manner. Flow cytometry analysis indicates that the anti-proliferative effects of artesunate on MCF-7 cells stem from apoptosis. Furthermore, caspase colorimetric assays demonstrate a substantial increase in cellular levels of caspase-8, caspase-9, and caspase-3 in artesunate-treated cells. In summary, these findings suggest that artesunate inhibits MCF-7 breast cancer cell growth by inducing apoptosis through intrinsic and extrinsic caspase-dependent pathways. Therefore, artesunate emerges as a promising candidate for breast cancer treatment.

In a study by Masoud V *et al.*, 2017, the author concluded that the primary obstacle in current breast cancer research lies in overcoming resistance mechanisms to targeted therapies. Combining various molecules to target multiple levels of signaling pathways, thus synergistically obstructing cancer cell escape routes and minimizing the development of survival mechanisms, holds promise for future advancements. It's crucial to conduct specific molecular profiling, especially for metastatic relapses, to better understand resistance phenotypes and facilitate the identification of new targets. Numerous ongoing clinical trials aim to enhance survival outcomes, particularly for challenging cases.

2.3 CYCLO(-Pro-Tyr)

In essence the article by Bojarska J *et al.*, 2021 states that, cyclic dipeptides (DKP) offer diverse structural and biological potential. Recent research has unveiled numerous DKPs and their derivatives from natural sources, showcasing novel bio-functionalities. Proline-based DKPs serve as valuable molecular scaffolds, adaptable for applications in synthetic biology and protein engineering. By manipulating additional amino acids and stereochemistry, these molecules can be tailored for various purposes, with potential for further chemical modifications to enhance their bio-activity. This overview synthesizes recent scientific and patent literature, offering insights into proline-based DKPs and their supramolecular structuring. With their compact and stable nature, these molecules facilitate the creation of rigid and biomimetic self-assemblies, ideal for addressing complex biological challenges. Such advancements hold promise for innovative biomedical and biotechnological applications, particularly in personalized medicine. Overall, this guide aims to deepen understanding of proline-containing DKPs and inspire further exploration in drug discovery, utilizing them as versatile scaffolds for designing smart therapeutics and bio-functional materials.

In an article by Karanam G *et al.*, 2020, the discovery of the anticancer properties of the cyclo dipeptide (DP) isolated from marine sponge *Callyspongia fistularis* associated *Bacillus pumilus* AMK1 has shed light on potential novel therapeutic strategies for hepatocellular carcinoma (HepG2) cells. Through comprehensive investigations, it was demonstrated that DP induces apoptosis in HepG2 cells primarily by disrupting mitochondrial function and suppressing the PI3K/Akt signaling pathway in a reactive oxygen species (ROS)-dependent manner. The activation of caspase-3, release of cytochrome c from mitochondria into the cytosol, and modulation of Bcl-2 family proteins further corroborated the apoptotic mechanism triggered by DP. Notably, DP treatment led to a decrease in phosphorylated PI3K and AKT levels, while enhancing PTEN expression, indicating a downregulation of the PI3K/AKT pathway. These findings underscore the potential of DP as a promising therapeutic agent for hepatocellular carcinoma, offering insights into its mechanistic actions and highlighting

its significance as a targeted therapy. However, further investigations, particularly in experimental in vivo models, are warranted to fully elucidate the anticancer efficacy and apoptosis-inducing activity of DP, thereby paving the way for the development of innovative therapeutic interventions against hepatocellular carcinoma.

A study by Karanam *et al.* 2020, demonstrated that cyclo(-Pro-Tyr), a novel dipeptide derived from the marine sponge-associated *Bacillus pumilus* AMK1, exhibits significant anticancer effects on HepG2 liver cancer cells. The molecule was shown to inhibit cell growth and initiate apoptosis through G2/M phase cell cycle arrest. These findings suggest that cyclo(-Pro-Tyr) has potential therapeutic implications for the treatment of hepatocellular carcinoma. The unique mechanism of action observed in this study underscores the value of marine-derived substances in the development of new anticancer agents. Further studies are needed to elucidate the detailed mechanisms behind the anticancer activities of cyclo(-Pro-Tyr) and to explore its potential in clinical applications.

2.4 DRUG-INDUCED APOPTOSIS OF CANCER CELLS

In an article by Karanam G *et al.*, 2020 it was summarized that the successful synthesis of ZnO nanoparticles (ZnONP) and their conjugation with dipeptide (DP) was achieved. Characterization using XRD, FESEM, and FTIR spectroscopy confirmed the formation of ZnONP-DP. Previous studies showed potent cytotoxic activity of DP against HepG2 liver cancer cells by inducing cell cycle arrest. In this study, ZnONP-DP was developed to enhance anticancer activity. Antiproliferative assays revealed that ZnONP-DP treatment effectively killed HepG2 cells compared to DP alone, with increased apoptosis. ZnONP-DP exhibited significant anticancer activity compared to ZnONP and DP alone. Toxicity studies on zebrafish embryos indicated teratogenic effects of ZnONP-DP only at higher concentrations. These findings suggest that dipeptide conjugated with ZnO nanoparticles holds promise as a potent treatment for liver cancer, offering a valuable resource for combating aggressive cancer cells.

Liu K *et al.*, 2015 performed studies to explore different methods of detecting apoptosis. One of them being acridine orange staining of cells. The results indicate that employing dual staining with acridine orange and ethidium bromide offers an economical and convenient means of detecting apoptosis in tumor cells and evaluating tumor chemosensitivity in comparison to flow cytometry. This method presents a cost-effective alternative that is both accessible and straightforward, potentially simplifying the processes of apoptosis detection and chemosensitivity testing. By leveraging the distinct staining properties of acridine orange and ethidium bromide, this dual staining technique enables researchers to visualize apoptotic cells with enhanced clarity, providing valuable insights into cellular responses to chemotherapeutic agents. Overall, the study underscores the practicality and efficiency of dual acridine orange/ethidium bromide staining as a valuable tool for apoptosis detection and chemosensitivity testing in tumor cells, offering a viable alternative to more complex and costly methodologies such as flow cytometry.

Kielkopf CL *et al.*, 2021 expressed that proteins are typically analyzed through electrophoresis in polyacrylamide gels, ensuring their dissociation into individual polypeptide subunits and preventing aggregation. Sodium dodecyl sulfate (SDS), a common anionic detergent, is often combined with a reducing agent and heat to denature proteins prior to loading onto the gel. SDS binds to the polypeptides, imparting a negative charge and masking their intrinsic charge, with the amount bound being generally independent of sequence and proportional to molecular weight. This protocol outlines the preparation and running of SDS-PAGE gels, followed by staining with Coomassie Brilliant Blue to detect proteins. Finally, the stained SDS-PAGE gel can be imaged or preserved by drying. This experiment can be used to detect apoptosis in cells.

Grela E *et al.*, 2018 explored the use of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay for assessing the metabolic activity of living cells. It relies on the enzymatic reduction of a tetrazolium salt to formazan,

resulting in a purple-blue coloration that can be measured spectrophotometrically. Optimal conditions yield absorbance values directly proportional to the number of viable cells. Initially developed for eukaryotic cell lines, the MTT assay has been adapted for use with bacteria and fungi. However, due to limited understanding of the MTT reduction mechanism in bacterial cells, various protocols have emerged, leading to potential inaccuracies and misinterpretation of results. This article provides a comprehensive overview of the MTT assay's applicability in research, highlighting sources of error and interpretation challenges.

2.5 ROLE OF NRF2 IN BREAST CANCER CELLS

In an article by Kumar H *et al.*, 2022, the author concluded that Nrf2 plays a vital role in maintaining cellular redox balance, impacting both normal and cancerous cells. Its absence can lead to increased ROS production, causing DNA damage and tumor formation. Nrf2 directly regulates the expression of GSH, TXN, and NADPH, controlling ROS levels. Interestingly, during oxidative stress, Nrf2 not only regulates NADPH but can also be activated by ROS-generated NADPH oxidase. These findings suggest Nrf2's involvement as an oncogenic factor. Understanding these cellular events and signaling pathways could lead to the development of improved treatment options for breast cancer.

Ghareghomi S *et al.*, 2022, expressed that Nrf2, a pivotal transcription factor within cells, intricately interacts with specific transcription factors and critical signaling pathways, forming a complex network of cellular communication. Understanding the roles of both ROS and the Nrf2 pathway is crucial for deciphering cellular characteristics. Upregulation of Nrf2 in cancer cells contributes to their unlimited growth, proliferation, and chemotherapy resistance. Ongoing research aims to validate these findings. Modulating various pathways in cancer cells directly impacts their ability to proliferate, metastasize, and develop multidrug resistance, key factors in cancer progression. Targeting the Nrf2 pathway and increasing ROS levels in cancer cells could enhance chemotherapy sensitivity. Investigating different Nrf2 inhibitors across

various cancers and their mechanisms of action could lead to the development of more potent anticancer agents, potentially reducing drug resistance. Repurposing existing drugs to counteract drug resistance in cancer cells also holds promise for improving treatment efficacy.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To identify the anticancer effect of cyclo(-pro-tyr) against human breast cancer cell lines through analysis of cellular mechanisms.

3.2 OBJECTIVES

- To check the anti-proliferative effect of cyclo(-pro-tyr) on human breast cancer cell line.
- To analyze the apoptosis influence of cyclo(-pro-tyr) by apoptosis assay and staining.
- To study the gene expression (BCL2, BAX, NF-KB, NRF2, and CASP3) through RT PCR analysis.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

Dulbecco's modified Eagle's medium (DMEM), Bovine Serum (FBS), 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Propidium Iodide (PI), Acridine Orange (AO), and Ethidium bromide (EB) were purchased from hi-media (California, USA), Cyclo(-Pro-Tyr) (CPT). All other reagents were purchased from Sigma Aldrich (USA) and were of analytical grade.

4.2 *INVITRO* CELL LINE STUDIES

Human breast cancer cell line models cultivated *in vitro* are frequently employed in cancer pharmacogenomic studies. Their purpose is to identify new pathways associated with variations in drug response, aid in establishing pharmacogenomic hypotheses for further investigation, and predict clinical responses (Iorio F *et al.*, 2016, Garnett MJ *et al.*, 2012). In this study, C2C12, MCF-7 and MDA-MB-231 cell lines were used. These cell lines were purchased through The National Centre for Cell Science (NCCS), Pune. The C2C12 cell line is a mouse myoblast cell line commonly used as an *in vitro* model for studying muscle development, differentiation, and regeneration. It was derived from the C3H mouse strain and was initially isolated from satellite cells found in the skeletal muscle tissue (Yaffe D *et al.*, 1977). The MCF-7 cell line is a human breast cancer cell line originally derived from a metastatic site of breast adenocarcinoma. It is one of the most extensively studied breast cancer cell lines and is widely used as a model system for studying breast cancer biology, drug response, and therapeutic interventions (Soule HD *et al.*, 1990). The MDA-MB-231 cell line is a human breast cancer cell line derived from a pleural effusion of a patient with metastatic breast adenocarcinoma. It represents a triple-negative breast cancer (TNBC) subtype, lacking expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 receptor (Cailleau R *et al.*, 1974).

4.2.1 Cell line maintenance

The cell line was cultured in DMEM medium supplemented with 10% FBS, along with 100 U/mL ampicillin and 100µg/mL streptomycin. Cultures were maintained in T25 flasks at 37°C in a humidified atmosphere with 5% CO₂. Upon reaching confluence, cells formed a monolayer adherent to the flask surface. Subsequent passaging involved trypsinization, where the culture medium was aspirated, and cells were rinsed with 2ml of phosphate buffered saline (PBS) to remove residual medium and cellular debris. Following this, 1 ml of 0.05% trypsin was added and incubated at 37°C for three to five minutes. Gentle tapping of the dish facilitated cell separation to prevent over trypsinization. Neutralization of trypsin was achieved by adding 2 ml of medium, and the cell suspension was centrifuged at 900 rpm for three minutes at room temperature to remove the supernatant. The pellet was resuspended in 2 ml of DMEM medium with 10% FBS, divided into four sterile bottles at 0.5ml each, and topped up to 2 ml with DMEM media in each bottle.

Table 4.1 List of cell lines in this study

Cell line	Media used	Source of cell line
C2C12 Fibroblast cell line	DMEM with 10% FBS	National Centre for Cell Science (NCCS), Pune
MDA-MB-231 Breast cancer cell line	DMEM with 10% FBS	National Centre for Cell Science (NCCS), Pune
MCF-7 Breast cancer cell line	DMEM with 10% FBS	National Centre for Cell Science (NCCS), Pune

4.2.2 Experimental Design

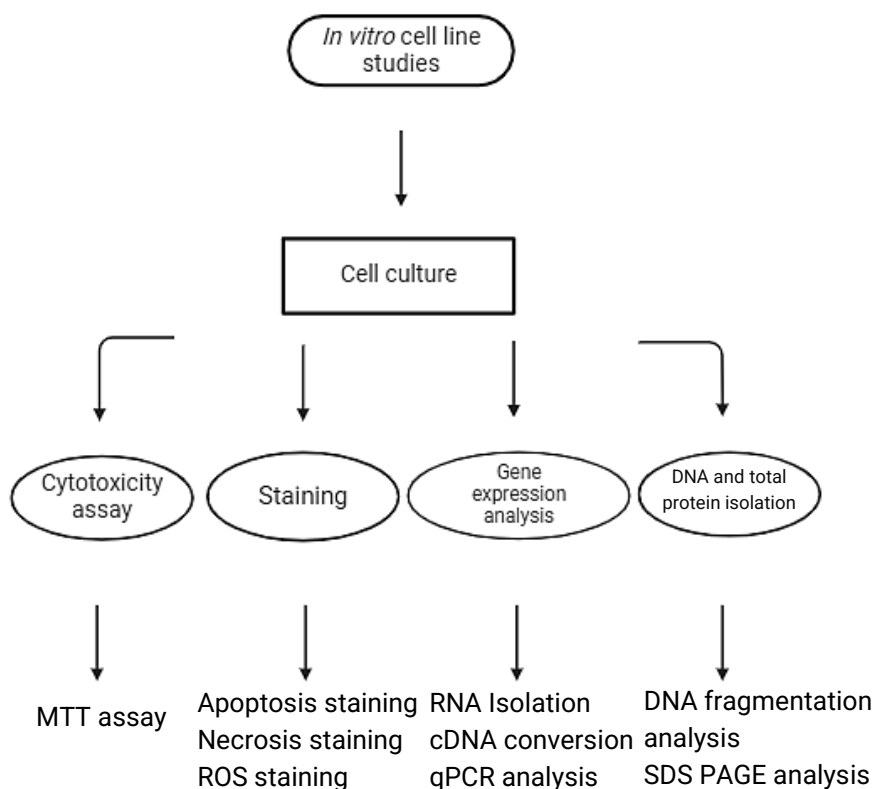


Fig 4.1 Experimental design of this study

4.2.3 Cell viability and cytotoxicity assay

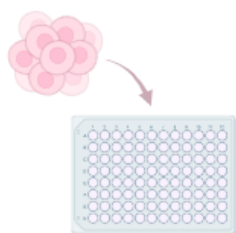
Cell viability was assessed using the MTT assay, investigating its growth-modulating effects on cultured breast cancer cell lines. Breast cancer cells (MDA-MB-231 and MCF-7) and non-cancerous fibroblast cells (C2C12) were seeded into 96-well plates and treated with varying concentrations of CPT for 24 hours. Following incubation, 50 μ L of MTT solution (1mg/mL) was added to the cells and incubated at 37°C for 4 hours. The insoluble formazan product was dissolved in DMSO, and MTT

reduction was quantified by measuring absorbance at 570 nm. The IC₅₀ value was determined as the concentration of extract causing a 50% inhibition of cell growth compared to control cells.

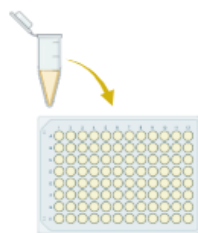
$$\text{Cell Viability (\%)} = \frac{\text{Mean OD}}{\text{control OD}} \times 100$$

MTT Assay

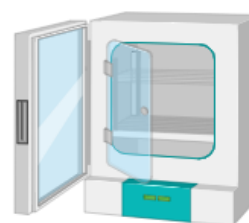
① Cell seeding in well plate



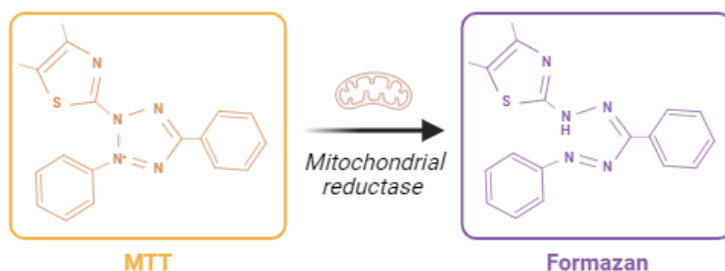
② MTT reagent addition



③ Incubation for 3-4 hours



④ Colorimetric reaction to determine cell viability



⑤ Absorbance reading (570nm)

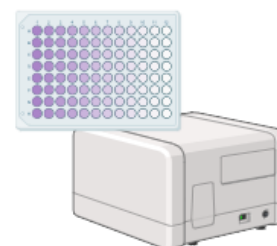


Fig 4.2 Cell viability assay

4.2.4 Necrosis assay by Propidium Iodine (PI) staining

The viability dye PI was utilized in an immunofluorescence microscopic analysis method to evaluate cell necrosis. This approach provides a quantitative assessment of the proportion of live and dead cells within a population through imaging. The assay

evaluates the integrity of the cell membrane, which becomes compromised during necrosis. Techniques such as PI staining or lactate dehydrogenase (LDH) release assays are employed to measure the release of cellular components, such as LDH enzyme or PI dye, from damaged cells into the surrounding medium. Real-time imaging techniques, such as time-lapse microscopy, enable the tracking of changes in cell viability associated with necrosis (Kondo, S *et al.*, 2005).

4.2.5 Apoptosis assay

Cell toxicity induced by CPT treatment was assessed based on its apoptosis-inducing properties. The apoptosis assay involved Acridine Orange/Ethidium Bromide (AO/EB) staining (Karanam G *et al.*, 2020). Cells were seeded in well plates, followed by treatment with the drug. After a specified duration, Acridine Orange and Ethidium Bromide (2 μ l of 1mg/ml each) were added to the plates. The mixture was then incubated at room temperature for 15 minutes. Real-time imaging techniques, such as time-lapse microscopy, were utilized to monitor changes in cell toxicity associated with apoptosis.

4.2.6 ROS staining

A fresh stock solution of DCFH-DA was prepared in sterile DMSO just before its use, ensuring avoidance of frozen and thawed dye. To remove any residual initial medium, the cells were washed with phosphate-buffered saline (PBS). The human hepatocarcinoma cell line was utilized in the experiment, cultured in standard media with reduced serum (2%), and treated with DCFH-DA at a final concentration of 1 μ M. The cultures were then incubated in a standard incubator set at 37°C with 5% CO₂ for 30 minutes in the dark. After removing the medium containing DCFH-DA, the cells were washed twice with PBS while being shielded from light. Fresh medium containing the preferred medication, in this case, H₂O₂ (0.03%), was added to the DCFH-DA loaded cells, and incubation continued as required. The cells were immediately analyzed using

fluorescence microscopy or a fluorescence plate reader equipped with the FL1 channel (green fluorescence) to determine the level of ROS. Fluorescence was observed using excitation and emission wavelengths suitable for green fluorescence (Karanam G et al., 2018).

4.2.7 RNA Isolation

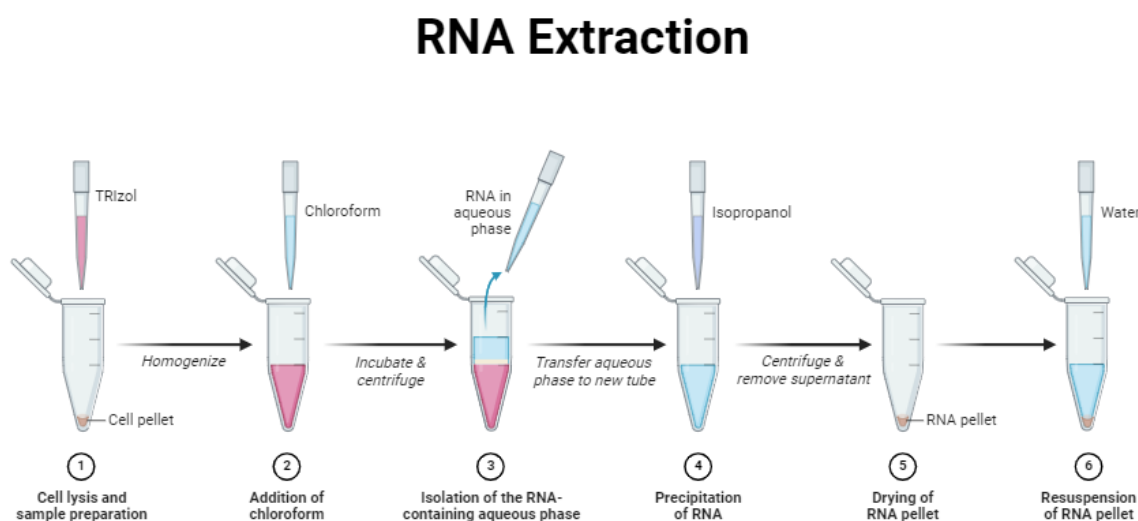


Fig 4.3 Schematic representation of RNA isolation

Isolating RNA from breast cancer cells requires careful attention due to their small size and susceptibility to RNA degradation. First, culture the breast cancer cells and maintain them in a well plate. Treat the cells with CPT at the IC₅₀ concentration and incubate for 24 hours. Aspirate the media and wash the cells once with 2ml of PBS, then remove the PBS. Add 1ml of Trizol to the cells, scrape them briefly, and transfer the Trizol to 2ml Eppendorf tubes. Allow it to sit at room temperature for 5 minutes, then add 250µl of chloroform and shake vigorously for 15 seconds. Let it sit for another 5 minutes, then centrifuge the samples to separate the phases. Transfer the aqueous phase containing RNA to a new tube, add isopropanol to precipitate the RNA, and centrifuge to pellet the RNA. Remove the supernatant, wash the RNA pellet with

ethanol, and air-dry or speed-vacuum to remove residual ethanol. Resuspend the RNA pellet in RNase-free water, optionally adding an RNase inhibitor. Finally, measure the RNA concentration and purity using a spectrophotometer (Rio DC *et al.*, 2010).

4.2.8 cDNA conversion

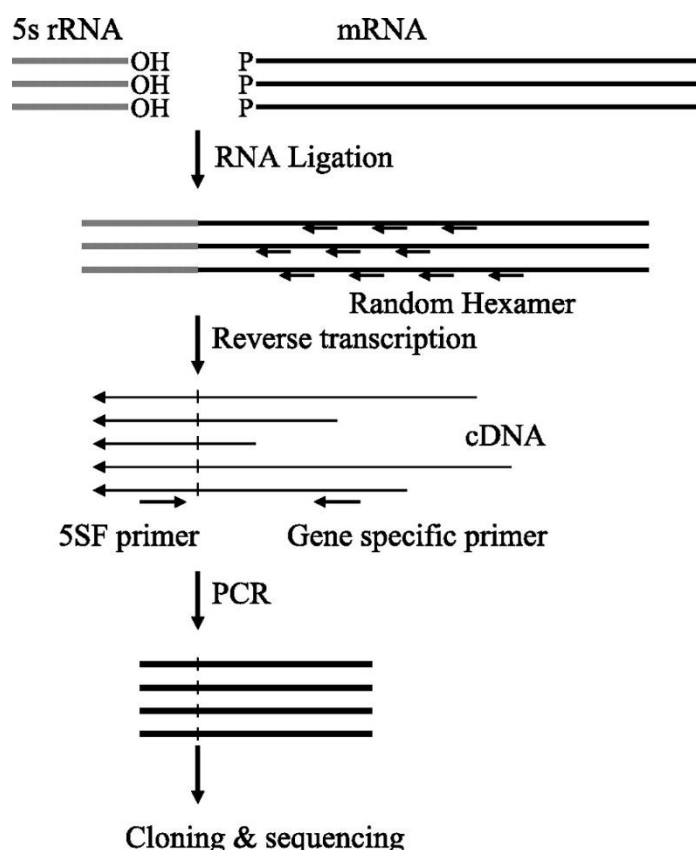


Fig 4.4 Representation of cDNA conversion (Lee HJ *et al.*, 2008)

Isolating cDNA from a specific compound like Cyclo(-Pro)(-Tyr) (CPT) involves a meticulous and step-by-step process. Initially, the CPT is extracted from the source material using extraction methods involving solvents, chromatography, or other purification techniques. Once the compound is extracted, RNA is isolated from the cells or tissues that produce CPT. Reverse transcription, performed using specialized kits, involves breaking down cell walls and membranes to release RNA, followed by the use

of an enzyme called reverse transcriptase to convert RNA into cDNA. This process, known as reverse transcription, generates complementary DNA strands from the RNA template. Subsequently, the cDNA is purified to remove any remaining RNA, enzymes, or impurities, which can be accomplished using purification kits or techniques like column purification, ethanol precipitation, or gel purification. Following purification, the cDNA is quantified to determine its concentration and assessed for quality using techniques such as spectrophotometry or gel electrophoresis.

4.2.9 Primer designing

Primer designing is a crucial step in laboratory procedures like polymerase chain reaction (PCR) and genomics. Primers are short, single-stranded DNA fragments that play a key role in these processes. By hybridizing with the sample DNA, two primers facilitate the PCR process, enabling the rapid generation of millions of copies of the target DNA. Additionally, primers are utilized in DNA sequencing and various other experimental procedures. We have used apoptosis gene expression (caspase-3, Bax and Bcl-2), inflammatory gene expression (NF- κ B), and antioxidant gene expression (NRF2) by qPCR analysis.

S.NO	TARGET GENE	FORWARD PRIMER	REVERSE PRIMER	Ta (°C)
1.	Bax	CTACAGGG TTTCATCC AG	CCAGTTCA TCTCCAA TTCG	55-60 °C
2.	BCL-2	GTGGATGA CTGAGTAC CT	CCAGGAGA AATCAAAC AGAG	55-60 °C
3.	Caspase-3	CATGGAAG CGAATCAA TGGACT	CTGTACCAG ACCGAGAT GTCA	55-60 °C
4.	NRF2	TCAGCGAC GGAAAGAG TATGA	CCACTGGTT TCTGACTGG ATGT	55-60 °C
5.	β -actin	CATGTACG TTGCTATCC AGGC	CTCCTTAAT GTCACGCA CGAT	55-60 °C

Table 4.2 Gene expression primers by qPCR analysis

4.2.10 RT-PCR analysis

RT-PCR (reverse transcription-polymerase chain reaction) has become a widely utilized variant of the PCR technique. Traditionally, RT-PCR involves two distinct steps:

the reverse transcription (RT) reaction and the subsequent PCR amplification. In the RT step, RNA is converted into complementary DNA (cDNA) using reverse transcriptase. The resulting cDNA serves as the template for the PCR amplification, wherein specific primers are used to amplify one or more target genes. RT-PCR offers the advantage of quantifying mRNA levels even from minute samples. Its sensitivity is such that it can accurately quantify RNA from a single cell.

4.2.11 DNA fragmentation analysis

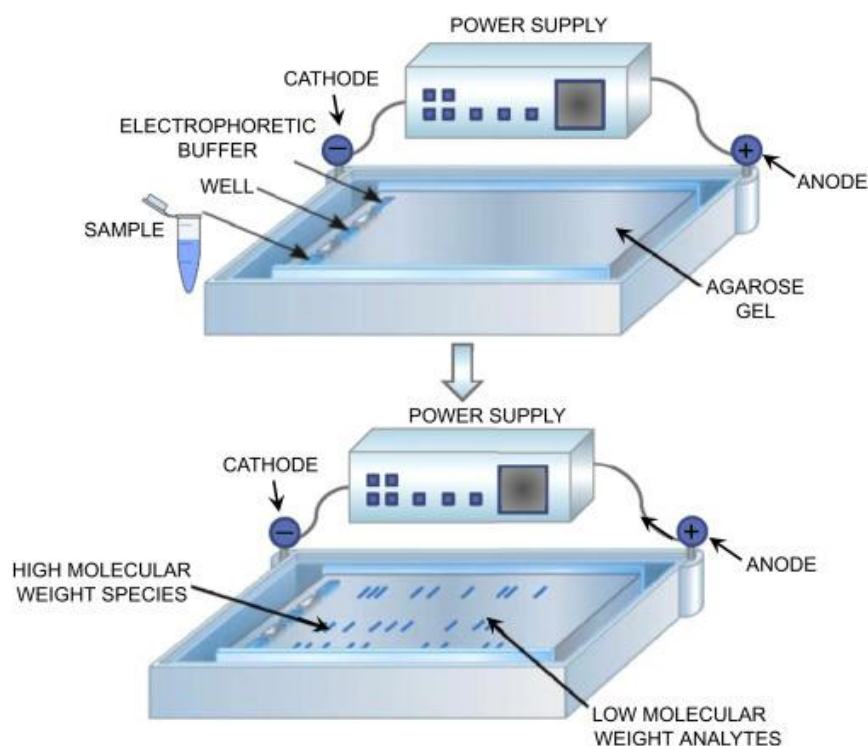


Fig 4.5 Schematic representation of Agarose Gel electrophoresis

DNA fragmentation analysis is performed by using Agarose gel electrophoresis. Agarose gel electrophoresis is a fundamental technique widely used to separate and analyze DNA fragments based on their size (Karanam G *et al.*, 2020). In this experiment, agarose gel electrophoresis was employed to visualize and analyze DNA

isolated from CPT treated breast cancer cells and normal breast cancer cells. The experiment began by preparing an agarose gel with a suitable concentration (1% agarose gel was used here) and casting it in a gel tray. Once the gel solidified, wells were formed at one end using a comb. The DNA samples, along with loading dye, were then loaded into the wells. When an electric current of 100 Volt was applied, the negatively charged DNA fragments migrated through the agarose matrix towards the positively charged electrode, with smaller fragments moving faster than larger ones. After electrophoresis, the gel was stained with a DNA-specific dye such as ethidium bromide and visualized under ultraviolet (UV) light.

4.2.12 SDS-PAGE analysis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), is a technique utilized for separating proteins extracted from cells. Initially, to isolate the proteins, cells are washed with PBS to eliminate any residual culture media or proteins. Subsequently, cold cell lysis buffer, supplemented with protease inhibitors, is added to lyse the cells, and the lysate is then collected after incubation on ice. The resolving gel, composed of a higher percentage acrylamide/bis-acrylamide, and the stacking gel, with a lower percentage acrylamide/bis-acrylamide, are prepared according to the desired protein size range. Gel cassettes are assembled, and the gel mixture is poured, followed by the insertion of a comb to create wells. After polymerization, protein lysates are mixed with loading buffer containing SDS and a reducing agent, heated to denature the proteins, and then loaded onto the gel along with molecular weight markers. Electrophoresis is conducted in an appropriate buffer, and after completion, the gel is stained with Coomassie Brilliant Blue (CBB) and Silver stain separately, to visualize the separated protein bands. Excess stain is removed by destaining, and the gel can be optionally imaged or photographed for documentation and analysis purposes.

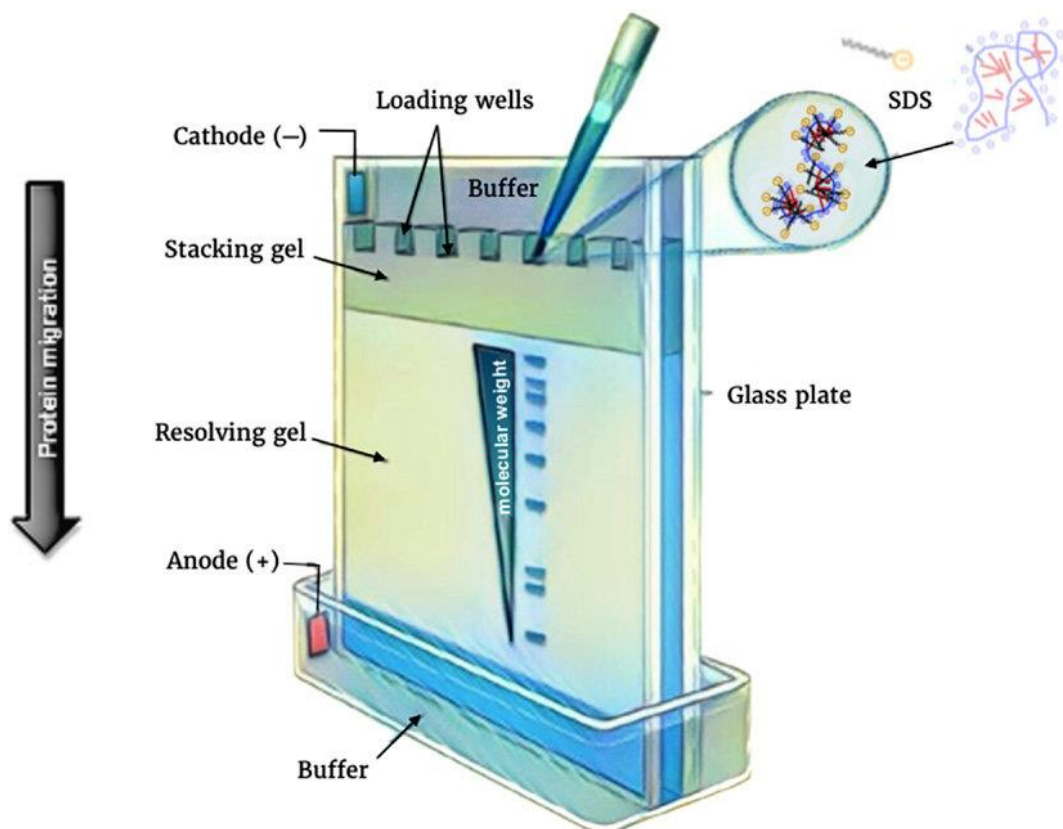


Fig 4.6 Schematic diagram of SDS-PAGE

CHAPTER 5

RESULTS AND DISCUSSION

5.1 CELL VIABILITY AND CYTOTOXICITY ASSAY

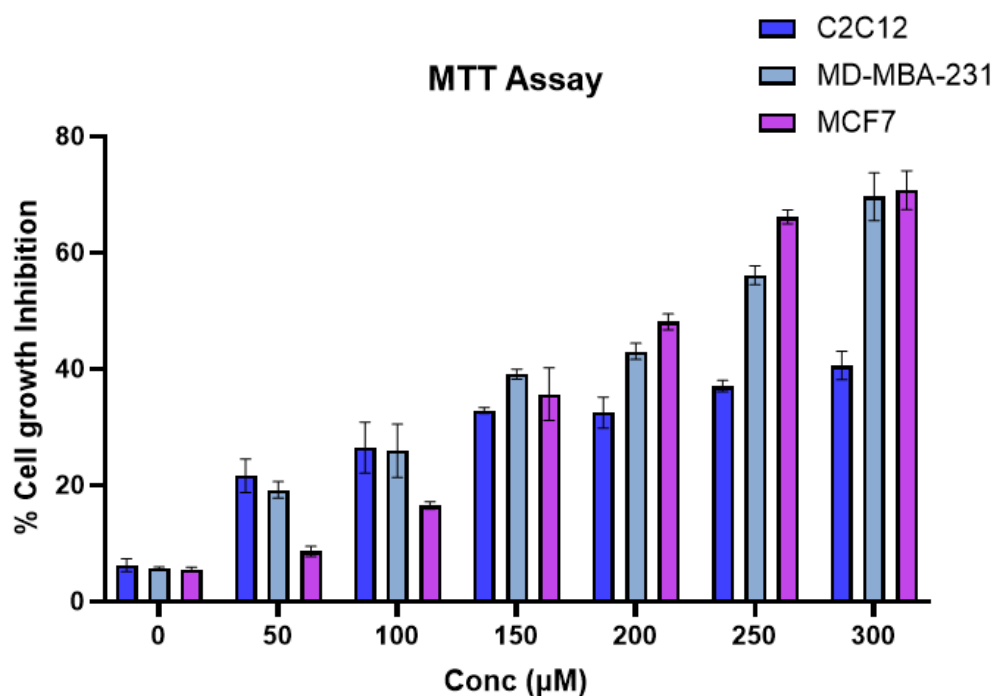


Fig 5.1 Viability of C2C12 (non-cancerous cell), MDA-MB-231(cancer cells), MCF 7 cells (cancer cells) after drug treatment at different concentrations (50, 100, 150, 200, and 300 µM) was estimated by MTT assay. Cell viability seem to decrease in both cancerous cell lines as the concentration of the drug increased. Data are represented as mean $\pm$ SD from (n=3) experiments.

The anti-proliferative and cytotoxicity effect of CPT on the growth of C2C12, MCF-7, MDA-MB-231 cells were initially determined by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). Cells were treated with increasing

concentrations of CPT (50 μ M, 100 μ M, 150 μ M, 200 μ M, 250 μ M, and 300 μ M) with DMEM media for 24h (Fig 5.1). CPT inhibited the growth of breast cancer cells in a dose dependent manner. IC50 value for each cell lines were determined through MTT data - 200 μ M (MCF-7), 230 μ M (MDA-MB-231). The IC50 value for C2C12 cells were never reached in this study. So it can be deduced that CPT does not show cytotoxic effects on non-cancerous cells up to concentration of 300 μ M. From these results from MTT assay, we have proceeded with other cell proliferation inhibition experiments on MDA-MB-231 cell line, which is a high metastatic breast cancer cell line.

5.2 CELL IMAGING

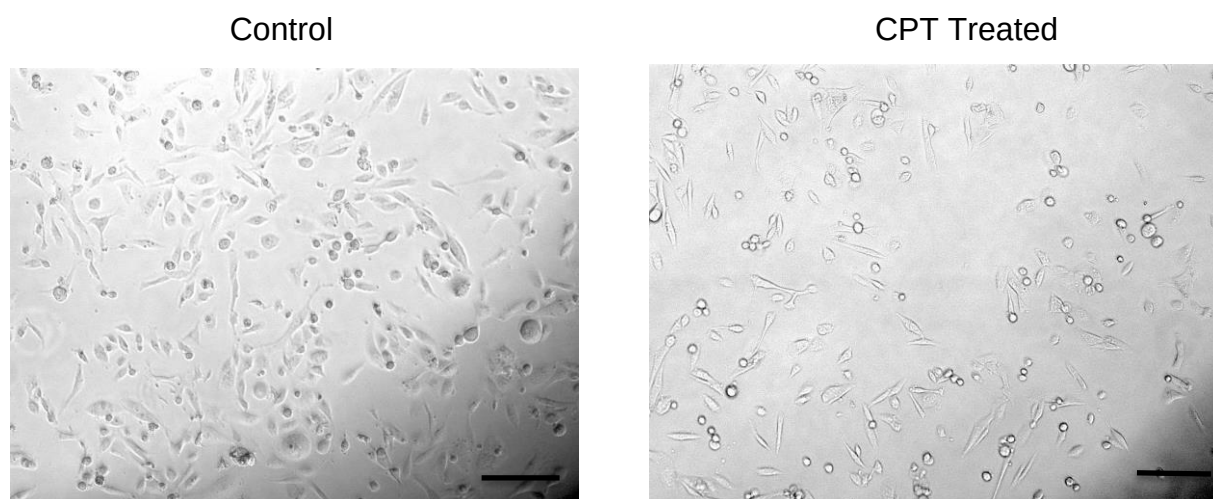


Fig 5.2 Phase contrast images of MDA-MB-231 cells showing morphological changes from CPT drug treatment. Prominent changes such as cell shrinkage, membrane blebbing, cell fragmentations and detachment mimicking apoptosis were observed at magnification 20x. (Scale balance = 50 μ m)

Fig 5.2 shows the morphological analysis of MDA-MB-231 high metastasis breast cancer cell line treated with IC50 concentration of CPT. Control picture shows normal cancer cell morphologies whereas CPT treatment showed shrunken cell

morphology with less population indicating apoptosis or cell death. Few cells showed membrane blebbing and chromatin condensation which confirms the anticancer effect of CPT for 24hrs treatment.

5.3 PI STAINING

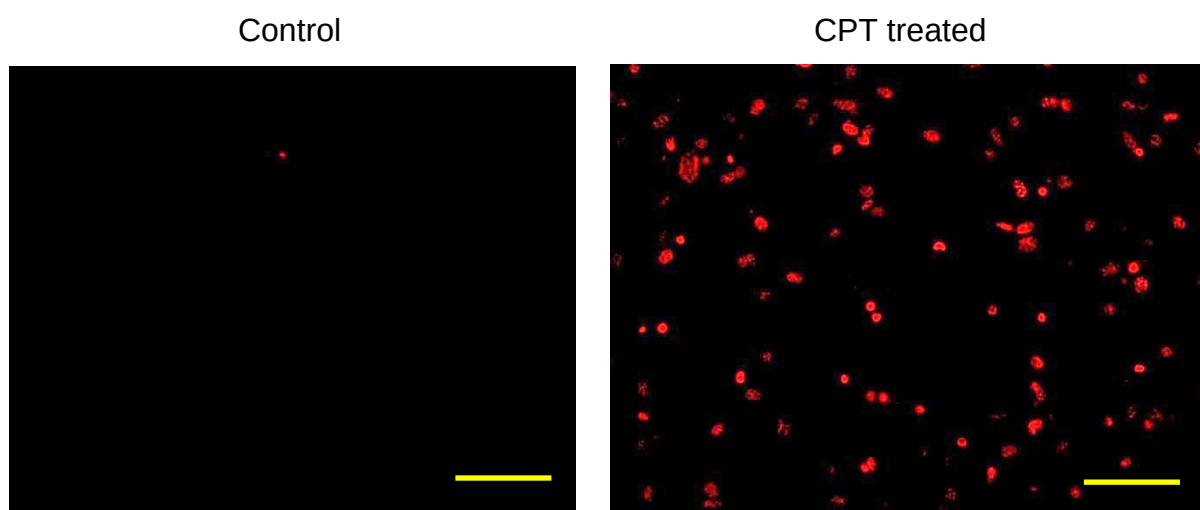


Fig 5.3 Propidium iodide (PI) staining of the MDA-MB-231 breast cancer cells, showing increase in PI-positive (red-stained) cells indicative of apoptosis. (Scale balance = 50 μ m)

PI staining is a valuable method for distinguishing between cell death mediated by apoptosis or necrosis. PI was used to study the apoptotic effect of CPT on MDA-MB-231 cells. Propidium iodide being fluorescent dye, cannot permeate through cell membrane. Therefore, dead cells would be stained fluorescent red (PI-positive cells). In fig 5.3, the control cells which were alive cells, did not show any red stained cell. The cells which were treated with CPT at IC50 concentration, show fluorescent red staining

which indicate necrotic cell death. On imaging the treated cells, cell fragmentation and detachment were observed.

5.4 AO/EB APOPTOSIS STAINING

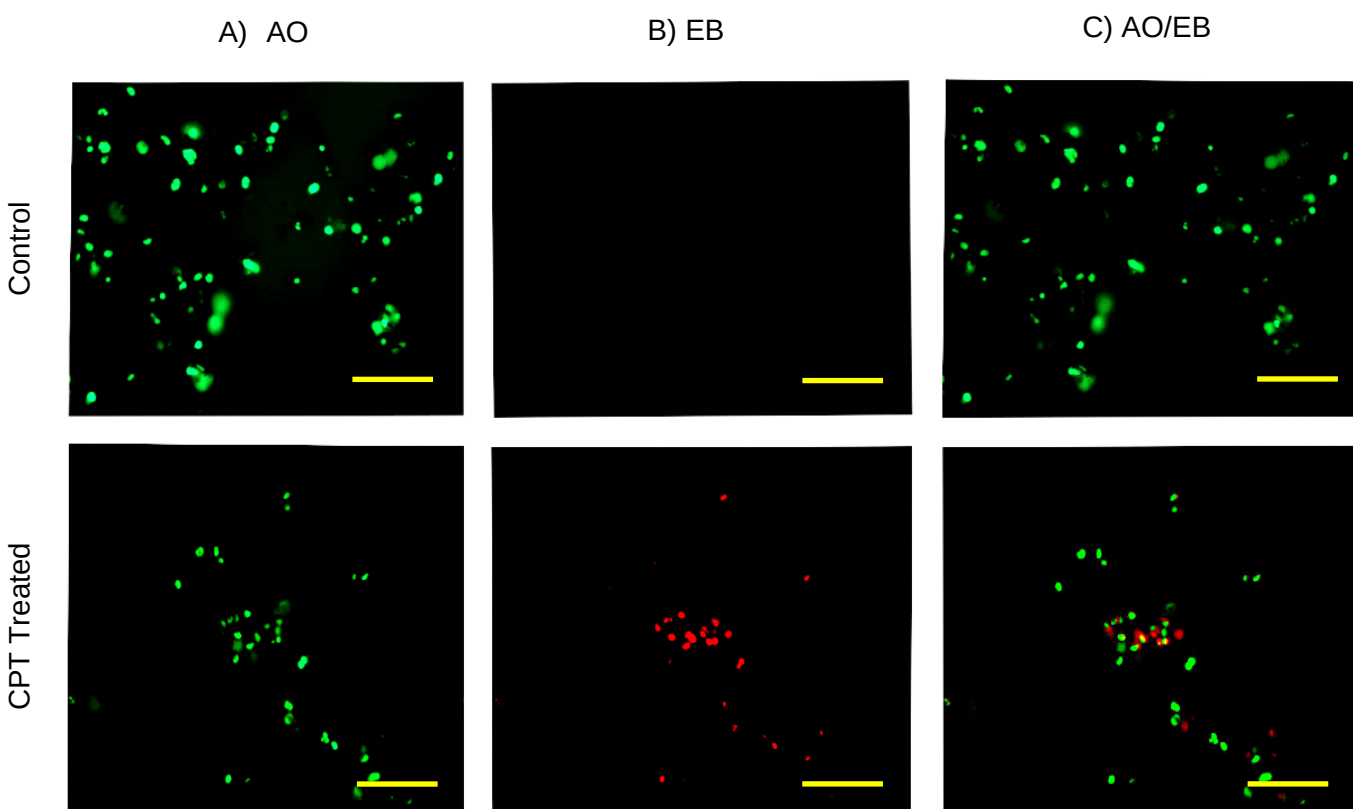


Fig 5.4 AO/EB staining of MDA-MB-231 cells. A) AO staining only (of alive cells); B) EB staining (of post-apoptotic cells); C) both AO and EB staining. (Scale balance = 50 μ m)

Acridine orange and ethidium bromide were used to study the cellular morphological alterations and apoptosis in CPT treated MDA-MB-231 cells. Apoptotic

cells were seen as the cells having membrane blebs and with apoptotic bodies. Acridine orange stains both the dead and live cells, whereas ethidium bromide stains only dead cells, as it cannot cross an intact plasma membrane. Through fig 5.4, it can be seen that control MDA-MB-231 cells produced green fluorescence with normal cytoplasm and nuclei morphology. Whereas, treated cells showed early apoptosis characterized by yellowish-green fluorescence with membrane and late apoptosis cells characterized by orange/red fluorescence. From this study, CPT showed significant apoptosis-inducing effect on liver cancer cells

5.5 DCFH-DA STAINING

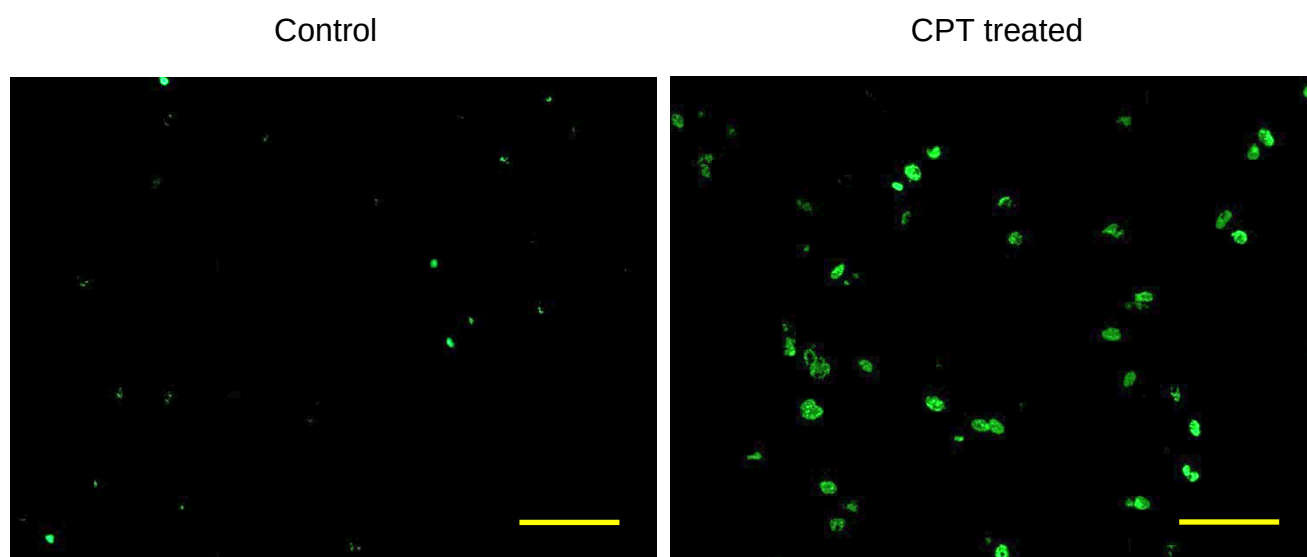
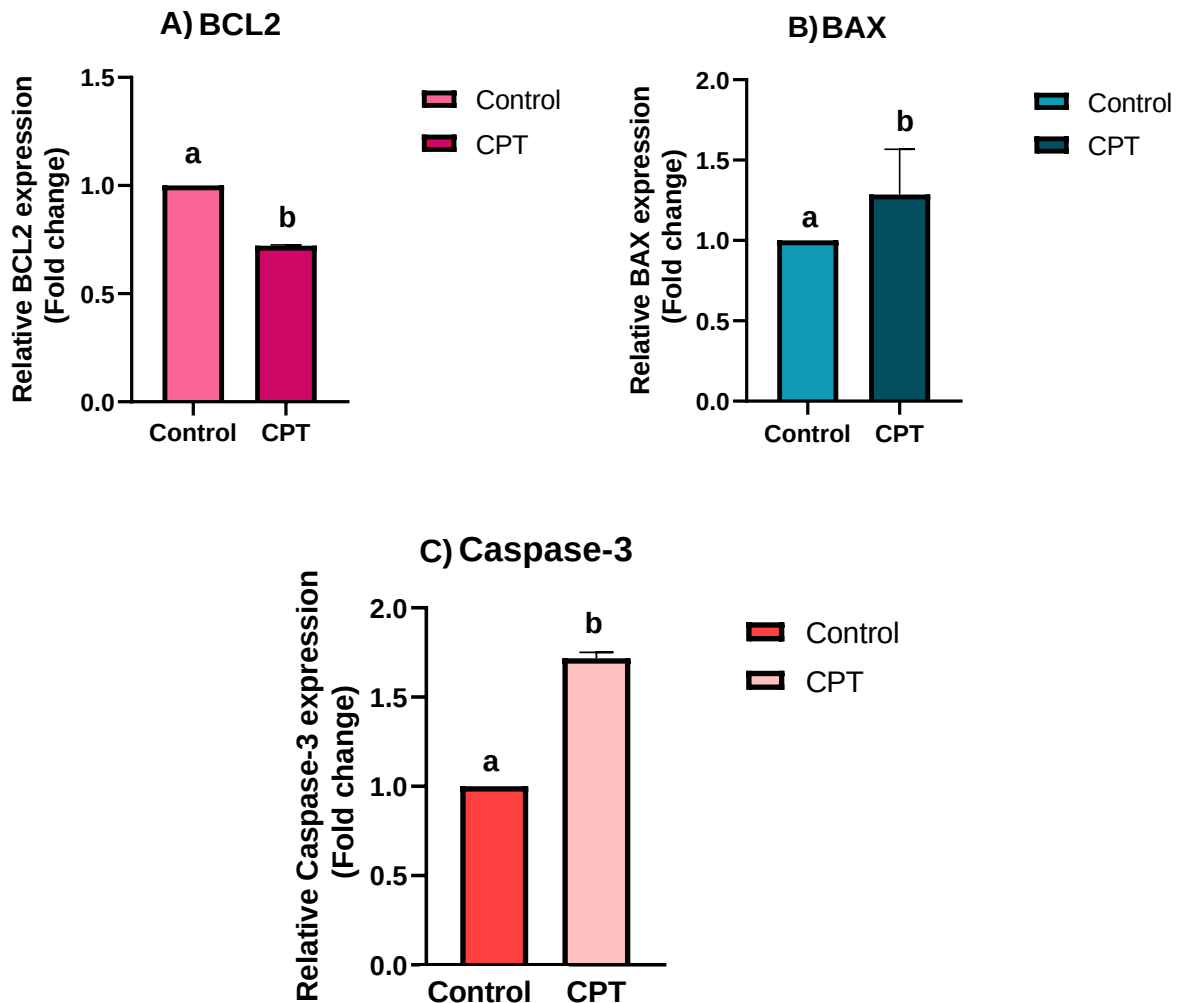


Fig 5.5 Fluorescence images (20x magnification) of DCFH-DA stained MDA-MB-231 cells. CPT treated cells stained with DCFH-DA showed increased fluorescence intensity of DCF (green) compared to untreated/control cells. (Scale balance = 50 μ m)

One of the most often methods of treating cancer is through chemotherapy. Chemotherapy works by increasing ROS generation in the cell using powerful

chemicals. It is hypothesized that chemotherapeutic amplification of ROS levels pushes the cancer cells over a threshold to induce cell death. To test if CPT induces the same, we perform DCFH-DA staining. The DCFH-DA fluorescent stain binds to ROS in the cell, giving it a green fluorescent colour. On observing the fig 5.5, it can be seen that control cells are not fully stained indicating the low level of ROS in the cell. The treated cell has high level of ROS detected by the fluorescent green stained cells.

5.6 RT-PCR ANALYSIS



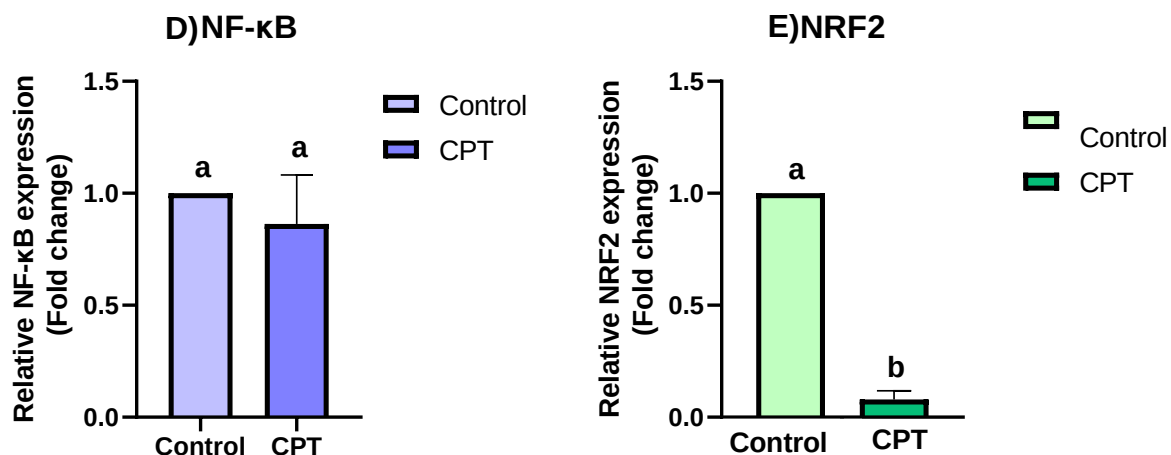


Fig 5.6 RT-PCR gene expression analysis of different gene markers: A - BCL2, B – BAX, C – CASPASE-3, D –NF-KB, E – NRF2. Data represented as mean $\pm$ SD from (n=3) experiments. Values represented as different letters are significant to each other.

qPCR analysis of Bcl2, Bax and Caspase-3 was analysed in MDA-MB-231 cells treated with CPT. In Fig 5.6, the mRNA expression level of Bcl2, Bax and Caspase-3 significantly altered during the treatment with CPT. The decreased mRNA level of anti-apoptotic gene Bcl2 and significant increase in the mRNA level of pro-apoptotic gene Bax and Caspase-3 at 24h treatment with CPT. This pattern of gene expression strongly supports with the protein expression of apoptosis inducing role of CPT through up-regulation of Bax and Caspase-3 while supporting the down regulation of Bcl2 expression. The inflammatory gene NF-KB expression was reduced. The antioxidant activity is technically controlled by NRF2 gene. In cancer cells, NRF2 expression is increased which supports the survival of the cancer cell. This gene expression was reduced indicating that CPT reduces the mortality of MDA-MB-231 cell at IC50 concentration.

5.7 DNA FRAGMENTATION ANALYSIS

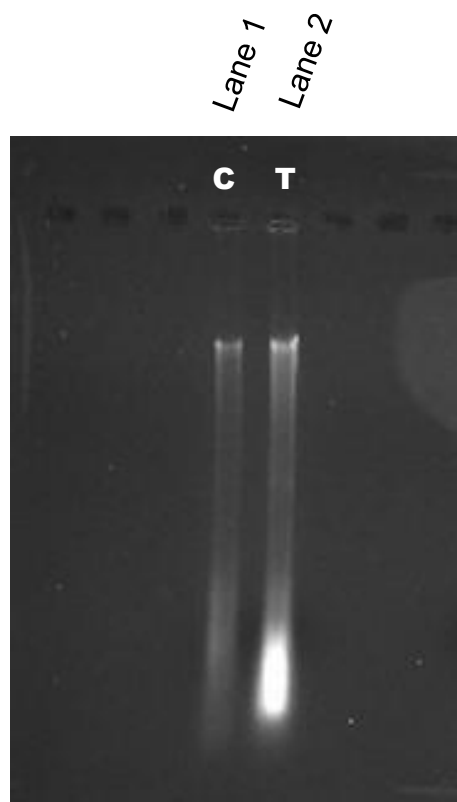


Fig 5.7 Visualization of DNA bands. Fragmentation of the DNA was visualized through agarose gel electrophoresis under UV light (Lane 1 - C – Control, Lane 2 - T – CPT Treated).

DNA fragmentation is one of the hallmarks of apoptosis. The activity of caspase-3 is closely related to the initiation of DNA fragmentation. Following activation of caspase-3 upon treatment with the extracts, the proteolytic cleavage of caspase-3 substrates is triggered. In Fig 5.7, we can see that the Lane 1 which contains genomic DNA of control MDA-MB-231 cells and Lane 2 has genomic DNA of treated MDA-MB-231 cells. On observation, it was seen that control cells have intact DNA and the treated cell's DNA were fragmented and accumulated at the end of the agarose gel due to the electric charge.

5.8 SDS-PAGE ANALYSIS

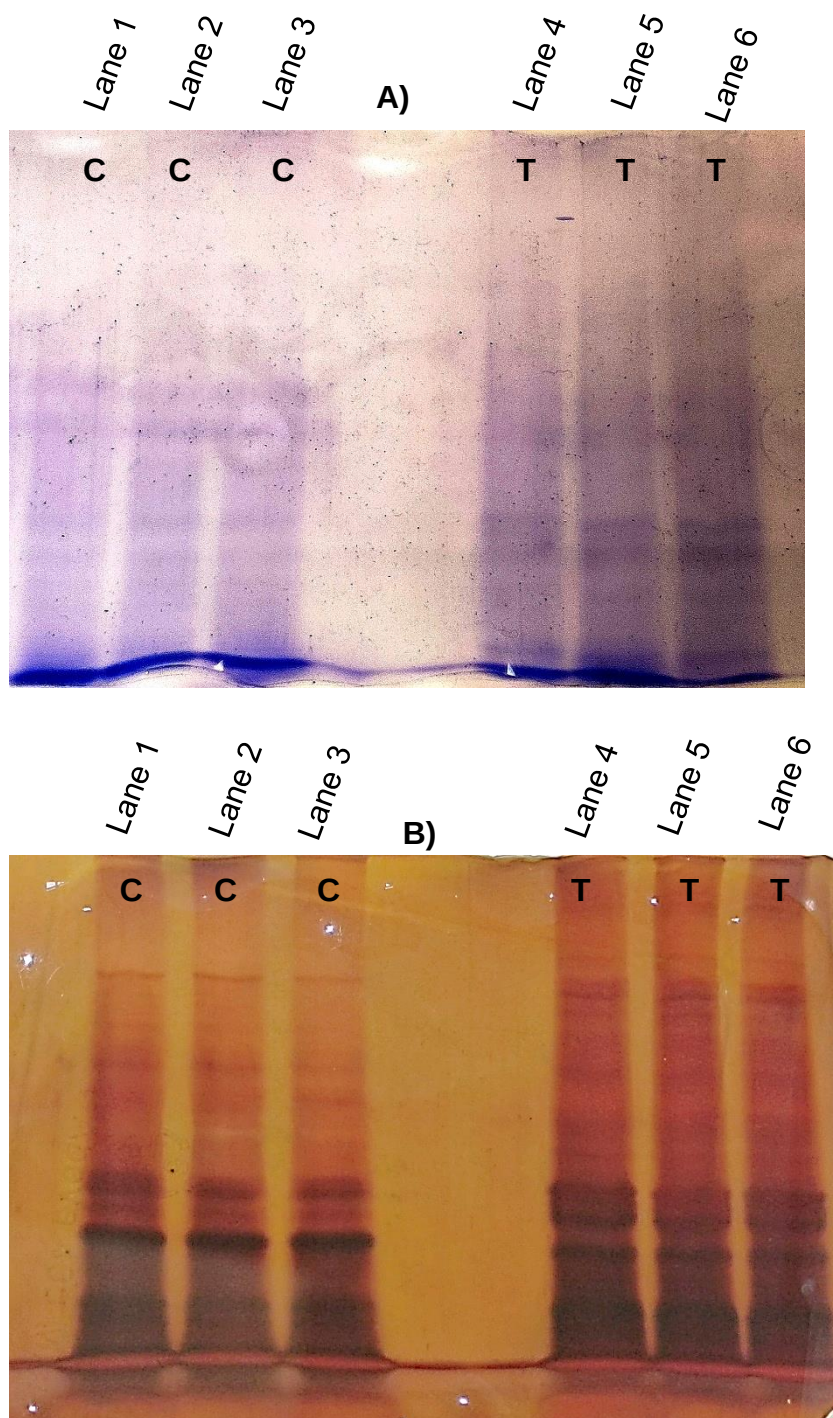


Fig 5.8 The SDS-PAGE gel stained image depicts separated protein bands, enabling analysis of protein size. (Lane 1, 2 & 3 – C - Control and Lane 4, 5, & 6 – T – Treated)

SDS-PAGE is used for analysis of protein expression in cells. The protein separates based on molecular weight of the protein. According to the Fig 5.8, the treated cells have increased total protein expression in comparison to control cells. The reasoning and explanation behind are yet to be studied in research by immunoblotting of the protein.

5.9 DISCUSSION

A detailed examination of cellular responses to cyclo(-Pro-Tyr) (CPT) treatment in MDA-MB-231 breast cancer cells was conducted, with an emphasis on apoptosis as the primary mechanism of action. The investigation began with the use of PI staining, a method known for its ability to identify apoptotic cells due to its intercalation into the DNA of membrane-compromised cells. The increased presence of PI-positive cells observed in the CPT-treated samples was significant, illustrating the compound's potential to compromise cell membranes and induce apoptosis(Nicoletti, I *et al.*, 1991). This outcome was critical as it demonstrated the potential of CPT as a therapeutic agent capable of targeting and eliminating cancer cells effectively. Further assessments were made using AO/EB staining techniques, which allowed for a more nuanced understanding of the stages of apoptosis. AO/EB staining are particularly effective in differentiating between viable, early apoptotic, and late apoptotic cells (Ribble, D. *et al.*, 2005). The analysis showed a clear shift towards apoptosis in CPT-treated cells, highlighting not only the increase in cell death but also the impact on overall cell viability. This dual-staining method contributed significantly to the understanding of how CPT disrupts cellular function. Additionally, the DCFH-DA staining method was employed to measure the intracellular production of reactive oxygen species (ROS), an important indicator of cellular oxidative stress, which can lead to apoptosis (Huang, Q *et al.*, 2012). The results from this staining showed a marked increase in fluorescence intensity in the CPT-treated cells compared to untreated controls. This increase suggested an elevated oxidative stress level due to CPT treatment, supporting the hypothesis that CPT's mechanism of inducing apoptosis involves the generation of oxidative stress within the cells.

Molecular analysis was also conducted through RT-PCR to quantify the expression levels of key apoptosis-related genes. The results indicated a significant downregulation of BCL2, a gene known for its role in cell survival, alongside an upregulation of BAX and Caspase-3, both of which are critical promoters of apoptosis (Livak, K. J. *et al.*, 2001). These molecular changes were consistent with the observed increases in apoptosis and supported the conclusion that CPT effectively activates apoptotic pathways. Together, these various methodologies provided a comprehensive overview of the apoptotic effects of CPT on MDA-MB-231 breast cancer cells, reinforcing its potential as an effective anti-cancer agent. The findings from these analyses contribute to the broader understanding of CPT's therapeutic mechanisms and offer valuable insights into its potential application in cancer treatment. The combination of cellular and molecular data elucidated the profound impact of CPT on inducing cell death, making a strong case for its further investigation and potential development into a treatment modality.

CHAPTER 6

SUMMARY AND CONCLUSION

Anti-proliferative effect can be attributed to altered biochemical mechanisms including suppression of cell proliferation, growth arrest at the checkpoints of cell cycle and enhanced apoptosis induction. The MTT assay revealed a significant dose-dependent decrease in cell viability upon treatment with cyclodipeptides (CPT), indicating their potent antiproliferative activity against human breast cancer cell lines. The progression from benign to malignant neoplasms is significantly influenced by apoptosis and its related cellular phenomena, making them crucial targets for cancer therapy across various malignancies. Staining for apoptosis by AO/EB revealed that CPT is able to induce apoptosis by early and late apoptosis. PI staining for necrosis or cell death and morphology change revealed that during CPT treatment the cell membrane damage, cell shrinkage and morphology change was observed. General chemotherapy drugs induce ROS generation in cancer cells which kills them. We estimated ROS generation in breast cancer cells by DCHF-DA staining where the fluorescent dye binds to the ROS.

The apoptosis pathway reliant on mitochondria entails the liberation of Cytochrome c from mitochondria into the cytosol. This occurs through either the inhibition of anti-apoptotic proteins or the stimulation of pro-apoptotic proteins within the Bcl-2 family, ultimately resulting in the activation of Caspase-3. Gene expression studies were done to show the drug upregulated pro-apoptotic gene BAX and down regulation of anti-apoptotic gene BCL2 with CPT treatment of human breast cancer cells. DNA fragmentation, a hall mark of apoptosis, is assessed using agarose gel electrophoresis. The separated bands of DNA observed allows us to evaluate the effectiveness of CPT on human breast cancer cell line. SDS-PAGE analysis explores how cyclodipeptide affects human breast cancer cells via NRF2 and mitochondrial apoptosis pathways. Revealing the underlying mechanisms, it sheds light on cyclodipeptide's anti-cancer properties, guiding future research in breast cancer treatment.

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