

# **COMBINATIONAL THERAPY OF MONACOLIN-x AND AXITINIB FOR EFFECTIVE TREATMENT AGAINST HUMAN BREAST CANCER CELL LINE**

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

By

**KATHIRVEL K (40230020)**



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

## **SATHYABAMA**

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

**Accredited with Grade “A++” by NAAC  
JEPPIAAR NAGAR, RAJIV GANDHI SALAI,  
CHENNAI – 600119**

**APRIL-2024**



# SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited A++ Grade by NAAC | 12B Status by UGC | Approved by AICTE

[www.sathyabama.ac.in](http://www.sathyabama.ac.in)

## DEPARTMENT OF BIOTECHNOLOGY

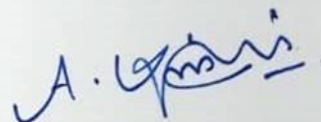
### BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of KATHIRVEL K (40230020) who carried out Project entitled "Combinational therapy of monacolin-x and axitinib for effective treatment against human breast cancer cell line" under my supervision from January 2024 to April 2024.

  
01/04/2024

Internal Guide

Ms. A. KAVI PRABA



External Guide

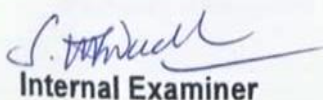
Dr. A. MADAN KUMAR



Head of the Department

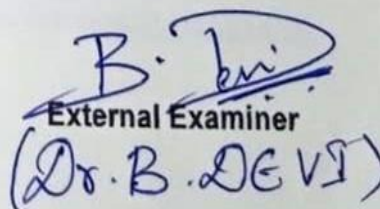
Dr. V. RAMESH KUMAR

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



Internal Examiner

(Dr. S. Ursha Nandhini)



External Examiner

(Dr. B. DEVI)

## **DECLARATION**

I, **Kathirvel.K (40230020)**, hereby declare that the Project Report entitled **“Combinational therapy of monacolin-x and axitinib for effective treatment against human breast cancer cell line”** done by me under the guidance of Ms. A. Kavi praba and Dr. A. Madan Kumar at the Centre for Molecular and Nanomedical Sciences, International Research Centre, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

**DATE:**

**PLACE: CHENNAI**

**SIGNATURE OF THE CANDIDATE**

## ACKNOWLEDGEMENT

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

I convey my thanks to **Dr. V. Ramesh Kumar, Ph.D.**, Head of the Department of Biotechnology for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Internal Project Guide **Ms. A. Kavi Praba, M.Tech.**, for her constant support and encouragement during the advancement of my project work.

I am extremely grateful to **Dr. A. Madan Kumar, Ph.D.**, Centre for molecular and nanomedical sciences, for his valuable guidance, suggestions, and constant encouragement paved way for the successful completion of my Project work.

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

## ABSTRACT

Breast cancer, a heterogeneous disease with diverse subtypes, poses a significant health challenge, necessitating innovative treatment approaches to improve patient outcomes especially for metastatic breast cancer or TNBC. While monotherapy treatments have been used for many years, they are not always effective, and many patients still relapse after initial treatment. Recently, combination therapies have emerged as promising new treatment options. In this present study, we investigated the combination of axitinib a potent inhibitor of tyrosine kinases involved in angiogenesis, and monacolin-x, a fungal secondary metabolite isolated from marine sponge with anti-cancer properties against MCF-7 and MDA-MB-231 cell line in-vitro and in-vivo. The research utilized various assays to assess the cytotoxicity, apoptosis induction, DPPH assay and DCFH-DA staining to measure antioxidant activity and intracellular ROS levels, and gene expression changes induced by the axitinib and monacolin-x combination. Furthermore, embryotoxic, teratogenic effects and ROS levels in zebrafish embryo were investigated. The MTT results revealed dose-dependent cytotoxicity in both MCF-7 and MDA-MB-231 cells with an IC<sub>50</sub> value of 82.7 $\mu$ M and 93.3 $\mu$ M respectively and showed synergistic effect. Axitinib and monacolin-x combination induced apoptosis, it was evident by PI and AO/EB staining, showed enhanced antioxidant activity, increased intracellular ROS levels and downregulated NF- $\kappa$ B, IL-6, TNF  $\alpha$ , IL-1 $\beta$  mRNA expressions through qRT-PCR analysis. The embryotoxic studies on zebrafish embryos revealed the axitinib and monacolin-x combination at (10 $\mu$ g/ml+10 $\mu$ g/ml) was found to be less toxic with no such deformities and significantly inhibited ROS levels. Therefore, axitinib and monacolin-x combination has potent anti-cancer activity against TNBC cell line MDA-MB-23 and further studies are needed to explore the synergistic effects of these combinations for clinical implications in breast cancer.

**Keywords:** Breast cancer, Combination therapy, Axitinib, Monacolin-x

## TABLE OF CONTENTS

| CHAPTER<br>NO | CONTENTS                                                   | PAGE NO  |
|---------------|------------------------------------------------------------|----------|
|               | Abstract                                                   | iv       |
|               | List of Abbreviations                                      | x        |
|               | List of Figures                                            | xi       |
|               | List of Tables                                             | xii      |
| <b>1.</b>     | <b>INTRODUCTION</b>                                        | <b>1</b> |
| 1.1           | Cancer                                                     | 1        |
| 1.2           | Breast Cancer                                              | 1        |
| 1.3           | Breast Cancer Therapeutics and Challenges                  | 3        |
| 1.4           | Combination Therapy for Treatment of Breast cancer         | 5        |
| 1.5           | Tyrosine Kinase Inhibitors: A New Hope in Cancer treatment | 6        |
| <b>2.</b>     | <b>REVIEW OF LITERATURE</b>                                | <b>8</b> |
| 2.1           | Combination therapy in cancer                              | 8        |
| 2.1.1         | Combination therapy in breast cancer                       | 9        |
| 2.2           | Tyrosine kinase inhibitors in breast cancer                | 9        |

|           |                                                                                          |           |
|-----------|------------------------------------------------------------------------------------------|-----------|
| 2.3       | Role of Axitinib in cancer                                                               | 10        |
| 2.3.1     | Role of Axitinib in breast cancer                                                        | 12        |
| 2.4       | Role of Monacolin-x in cancer                                                            | 13        |
| 2.5       | Role of TNF- $\alpha$ in breast cancer                                                   | 14        |
| 2.6       | Role of IL-6 in breast cancer                                                            | 14        |
| 2.7       | Role of IL-1 $\beta$ in breast cancer                                                    | 15        |
| 2.8       | Role of NF-kB in breast cancer                                                           | 15        |
| <b>3.</b> | <b>AIM AND OBJECTIVE</b>                                                                 | <b>16</b> |
| <b>4.</b> | <b>MATERIALS AND METHODS</b>                                                             | <b>17</b> |
| 4.1       | Chemicals and Reagents                                                                   | 17        |
| 4.2       | IN VITRO STUDIES                                                                         | 17        |
| 4.2.1     | Cell line and Culture                                                                    | 17        |
| 4.2.2     | Preparation of the Sample                                                                | 18        |
| 4.2.3     | Evaluation of growth inhibition and cytotoxicity of Axitinib and Monacolin-x combination | 18        |
| 4.2.3.1   | Evaluation of Axitinib and Monacolin-x combination effects                               | 20        |

|         |                                                                                           |    |
|---------|-------------------------------------------------------------------------------------------|----|
| 4.2.4   | Detection of Cellular Apoptosis                                                           | 20 |
| 4.2.4.1 | Propidium iodide (PI) staining                                                            | 20 |
| 4.2.4.2 | Acridine Orange (AO) / Ethidium Bromide (EB) staining                                     | 21 |
| 4.2.5   | Measurement of intracellular reactive oxygen species (ROS)                                | 22 |
| 4.2.6   | Evaluation of Antioxidant activity of Axitinib and Monacolin-x combination                | 23 |
| 4.2.7   | Total RNA Isolation                                                                       | 23 |
| 4.2.8   | cDNA Conversion                                                                           | 24 |
| 4.2.9   | Designing Primers for quantitative Real-Time-Polymerase Chain Reaction                    | 25 |
| 4.2.10  | Gene Expression analysis using Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) | 26 |
| 4.3     | IN VIVO STUDIES                                                                           | 27 |
| 4.3.1   | Maintenance and Spawning of Zebrafish ( <i>Danio Rerio</i> )                              | 27 |
| 4.3.2   | Zebrafish Embryo toxicity and teratogenicity Assay                                        | 27 |
| 4.3.3   | Measurement of ROS generation in zebrafish                                                | 29 |
| 4.4     | Statistical Analysis                                                                      | 29 |

|           |                                                                                                           |           |
|-----------|-----------------------------------------------------------------------------------------------------------|-----------|
| <b>5.</b> | <b>RESULTS AND DISCUSSION</b>                                                                             | <b>31</b> |
| 5.1       | IN VITRO STUDIES                                                                                          | 31        |
| 5.1.1     | Cytotoxicity effect of Axitinib and Monacolin-x combination treatment                                     | 31        |
| 5.1.1.1   | Combination effect of Axitinib and Monacolin-x                                                            | 34        |
| 5.1.2     | Evaluation of apoptosis induced by axitinib and monacolin-x combination treatment                         | 35        |
| 5.1.2.1   | Propidium Iodide (PI) Staining                                                                            | 35        |
| 5.1.2.2   | Acridine Orange and Ethidium Bromide Dual Staining (AO/EB)                                                | 37        |
| 5.1.3     | Axitinib and Monacolin-x combination treatment modulates intracellular ROS production in MDA-MB-231 cells | 39        |
| 5.1.4     | DPPH free radical scavenging assay                                                                        | 41        |
| 5.1.5     | Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)                                                | 42        |
| 5.2       | IN VIVO STUDIES                                                                                           | 45        |
| 5.2.1     | Embryo Toxic Effect of Axitinib and Monacolin-x on Zebrafish Embryos                                      | 45        |
| 5.2.1.1   | Effect of Axitinib and Monacolin-x on Zebrafish Embryos Heartbeat Rate                                    | 47        |

|           |                                                                       |           |
|-----------|-----------------------------------------------------------------------|-----------|
| 5.2.2     | Teratogenic activity of Axitinib and Monacolin-x on zebrafish embryos | 48        |
| 5.2.3     | ROS level in zebrafish larvae                                         | 51        |
| <b>6.</b> | <b>SUMMARY AND CONCLUSION</b>                                         | <b>56</b> |
|           | <b>REFERENCES</b>                                                     | <b>58</b> |
|           | <b>ANNEXURE</b>                                                       | <b>68</b> |

## LIST OF ABBREVIATIONS

AO - Acridine Orange

BC - Breast Cancer

DCFH-DA - 2', 7'-dichlorodihydrofluorescein diacetate

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl Sulfoxide

DPPH - 2, 2-diphenyl-1-picrylhydrazyl

EB - Ethidium Bromide

FBS - Fetal Bovine Serum

IL-1 $\beta$  - Interleukin-1 beta

IL-6 - Interleukin-6

NF- $\kappa$ B - Nuclear factor-kappa B

PBS - Phosphate Buffered Saline

PI - Propidium Iodide

ROS - Reactive Oxygen Species

TKI - Tyrosine Kinase Inhibitor

TNBC - Triple Negative Breast Cancer

TNF- $\alpha$  - Tumor necrosis factor alpha

VEGFR – Vascular Endothelial growth Factor Receptor

## LIST OF FIGURES

| FIGURE NO | FIGURE NAME                                                                                              | PAGE NO |
|-----------|----------------------------------------------------------------------------------------------------------|---------|
| 5.1       | Percentage inhibition on MCF-7 and MDA-MB-231 cells.                                                     | 32      |
| 5.2       | Fa vs CI plot of MCF-7 and MDA-MB-231 cells.                                                             | 35      |
| 5.3       | Propidium Iodide stainig                                                                                 | 36      |
| 5.4       | Acridine Orange and Ethidium Bromide dual staining                                                       | 38      |
| 5.5       | Evaluation of intracellular ROS by DCFH-DA assay                                                         | 40      |
| 5.6       | DPPH free radical scavenging activity                                                                    | 42      |
| 5.7       | Relative mRNA expression of genes TNF- $\alpha$ , IL-6, IL-1 $\beta$ and NF- $\kappa$ B                  | 44      |
| 5.8       | Percentage mortality and hatchability of zebrafish embryos                                               | 46      |
| 5.9       | Heartbeat rate of the zebrafish embryos                                                                  | 47      |
| 5.10      | Effect of Axitinib and Monacolin-x as individual and combination on the development of zebrafish embryos | 49      |
| 5.11      | ROS production in zebrafish larvae                                                                       | 52      |

## LIST OF TABLES

| TABLE NO | TABLE NAME                                | PAGE NO |
|----------|-------------------------------------------|---------|
| 4.1      | List of cell lines                        | 18      |
| 4.2      | List of Primers used for qRT-PCR analysis | 26      |
| 4.3      | statistical analyses for experiments      | 30      |
| 5.1      | Fa and CI data                            | 34      |
| 5.2      | Fold changes in target genes              | 43      |

# CHAPTER 1

## INTRODUCTION

### 1.1 CANCER

Cancer is a complex and multifaceted disease characterized by uncontrolled growth and spread of abnormal cells in the body. Normal cells in our body have specific functions and a regulated life cycle, but cancer cells lose this control mechanism. They continue to divide and proliferate uncontrollably, forming tumors or invading surrounding tissues and organs. Cancer can develop in almost any part of the body, and it can originate from various types of cells. The transformation of healthy cells into cancer cells typically involves genetic mutations that disrupt the cell's ability to regulate growth and division (Robado de Lope *et al.*, 2023). These mutations can be caused by a variety of factors, including exposure to carcinogens, genetic predisposition, unhealthy lifestyle choices, and aging. As cancer progresses, it can interfere with the body's normal functions, leading to symptoms such as unexplained weight loss, fatigue, pain, and changes in bodily functions. Cancer is a major global health concern, impacting millions of people each year and posing significant challenges in terms of diagnosis, treatment, and management (Anand *et al.*, 2008). Research efforts continue to focus on understanding the underlying mechanisms of cancer development, identifying new treatment approaches, and improving outcomes for individuals affected by this complex disease. There are over 200 different types of cancer, each classified based on the specific cells they originate from and their distinct characteristics. The most common types of cancer in males are lung cancer, prostate cancer, colorectal cancer, and stomach cancer. In females, the most common types are breast cancer, colorectal cancer, lung cancer, and cervical cancer (<https://www.cancer.gov/>).

### 1.2 BREAST CANCER

Breast Cancer (BC) is a heterogeneous disease it begins when healthy cells in the breast undergoes changes or mutation and grows out of control, forming a mass or sheet of cells called a tumor. BC is the most common and malignant tumor diagnosed in females around worldwide and BC is a leading cause of cancer mortality among females according to GLOBOCAN reports (Sung *et al.*,

2021). In 2022, about 2.3 million females were diagnosed with BC worldwide and 670,000 were died. Every 14 seconds, somewhere in the world, a woman is diagnosed with BC. In upcoming years, BC is likely to increase over 3 million new cases and 1 million deaths by 2040 and BC in females has completely surpassed lung cancer as the leading cause of cancer incidence worldwide. Globally, BC now represents one in four of all cancers in females and since 2008, BC incidence has increased by more than 20 percent and Mortality has increased by 14 percent (Arnold *et al.*, 2022). In India, BC is the most common cancer accounting for 28.2% of all female cancers, with an estimated 216,108 cases (Mehrotra and Yadav, 2022). Despite advancements in early detection, approximately 30% of patients with early-stage of BC still relapse with comprehensive BC treatment. BC can be invasive or non-invasive. Invasive BC is cancer that spreads into surrounding tissues and/or distant organs. Non-invasive BC does not go beyond the milk ducts or lobules in the breast. About 80% of BC is invasive cancer, and about 20% is non-invasive cancer (Pescia *et al.*, 2023). BC is mostly developed by mutations or alterations in genes such as BRCA1, BRCA2, Tp53, PALB2, PTEN, MYC, PI3KCA, ERBB2 and GATA3. Mutation in these genes causes inherited and hereditary BC but it accounts only 5-10% of BC cases those results from genetic predisposition. Majority of BC are sporadic and are influenced by various risk factors that includes tobacco use, alcohol consumption, family predisposition, lack of physical activity, lactation inefficiency, imbalanced dietary habits, exposure to environmental toxins, delayed puberty, delayed menarche, delayed age of marriage, late menopause, hormone replacement therapy, use of contraceptive, and obesity (Kashyap *et al.*, 2022). BC are classified based mainly on the immunohistochemical expression of hormone receptors such as human epidermal growth factor receptor 2 (HER2) or (ERBB2), estrogen receptor (ER), and progesterone receptor (PR). More recently the technological advances have emerged, which classifies BC tumors into five distinct molecular subtypes (Luminal-A, Luminal-B, HER2-enriched, Basal-like, Non basal-like) based on mRNA expression (Turner *et al.*, 2021). Luminal-A subtype tumors are characterized by the existence of ER and PR and the lacking of HER2 receptors then Luminal-B subtype tumors are characterized by the existence of ER receptor and in some cases PR receptor and the lacking of HER2 receptor, both accounts for 60-70% of BC. HER2 enriched subtype tumors are characterized by the

existence of high HER2 receptor expression and the lacking of both ER and PR receptors that accounts for 12-20% of BC. Non basal-like and Basal-like subtype tumors are recognized as Triple-negative breast cancer (TNBC) subtypes that can be characterized by the lacking of HER2, ER and PR receptors that accounts for 10-15% of BC (Orrantia-Borunda *et al.*, 2022). Among these subtypes, TNBC is associated with aggressive tumor behavior, higher rates of metastasis and high recurrence rates with poor prognosis and it is particularly difficult to treat because of its lack of recognizable markers and hence the lack of specific druggable targets. Limited targeted treatment options for TNBC pose significant challenges in disease management (Lakshmaiah *et al.*, 2014). The overexpression of vascular endothelial growth factor receptors (VEGFR's) in TNBC has emerged as a potential therapeutic target, yet efforts to exploit this target have faced challenges, including the development of resistance mechanisms. Dysregulation of these pathways contributes to disease progression, angiogenesis, and resistance to treatment, highlighting their significance as potential therapeutic targets (Li *et al.*, 2021).

### 1.3 BREAST CANCER THERAPEUTICS AND CHALLENGES

Even though breast cancer is still very prevalent, there is a reduction in mortality over the years, mainly due to improvements in the diagnosis and management, varying widely between diverse geographic areas . However, the treatment of metastatic disease remains a huge challenge, despite advances with a better knowledge of the use of therapies for BC of early stage. In fact, metastasis is the major cause of mortality for BC patients, being the most severe form of the disease, in which a huge genetic complexity is present. Thus, the choices of treatment regimens for BC depend on diverse factors, namely the stage and subtype of cancer, the hormone receptor status, if the cancer is HER2 positive or negative, the overall health status of the person. These include surgery, endocrine therapy, radiation therapy, chemotherapy, immunotherapy, molecular specific targeted therapy and gene therapy (Trayes and Cokenakes, 2021). Surgery involves removal or excision of breast tumors by the procedure of lumpectomy; removal of the cancerous tissue or mastectomy; removal of the whole breast. Surgery is performed to remove the axillary lymph nodes to assess the cancers ability to spread by Sentinel lymph node biopsy (SLNB) or Axillary

lymph node dissection (ALND). Major challenge of breast surgery is their postoperative complications which includes seroma, haematoma, mastectomy flap necrosis, fat necrosis, venous thromboembolism, lymphedema, nerve injury and reduced shoulder mobility (Al-Hilli and Wilkerson, 2021). Radiation therapy involves using high-energy radiations in portions of breast before or after mastectomy and axillary surgery. BC exhibits Radiation therapy resistance which is the major challenge and it results in metastasis, recurrence and poor prognosis as they acquire resistance because of DNA damage repair, cell cycle redistribution, apoptosis escape, hypoxia-inducible factor 1 alpha protein (HIF-1 $\alpha$ ) in TME and cancer stem cells (CSC) (Burguin *et al.*, 2021). Endocrine therapy or Hormonal therapy involves in treatment of hormone receptor positive breast tumors such as ER<sup>+</sup> and PR<sup>+</sup> by utilizing Aromatase inhibitors and Selective estrogen receptor modulators (SERM) then the major challenge associated with hormonal therapy are acquiring resistance because of mutations in the ER gene Estrogen Receptor Alpha Gene 1 (ESR1) that leads to recurrence and metastasis (Herzog and Fuqua, 2022). Chemotherapy comprises of several drugs including anthracycline, taxanes and platinum based drugs for treatment of TNBC and HER2<sup>+</sup> receptor BC. Chemotherapy can be recommended for use in setting of neo-adjuvant chemotherapy; before surgery or adjuvant chemotherapy and after surgery. Major challenge for treatment of BC using chemotherapy is its resistance because of ATP-binding cassette (ABC) family member, mutations in microtubule-associated proteins (MAPs) (Kerr *et al.*, 2022). Immunotherapy involves in the treatment of TNBC by immune checkpoint inhibitors, cytokines, antibodies and chimeric antigen receptor-Therapy (CAR-T). Major challenge associated with immunotherapy is it works only in certain subset of patients because targets for TNBC are limited, intrinsic and extrinsic regulators are unknown. Genetic alterations in the genes involve in the development and progression of BC (Luo *et al.*, 2022). Gene therapy strategies such as gene replacement strategies, antisense strategy, RNAi therapy, suicide gene therapy are employed to down regulate the expression or replace the mutated genes and to silence unwanted gene expressions. The major challenge associated with genetic therapy is their effective delivery of the genetic material into the targeted site on cancer cells (Sheikh-Hosseini *et al.*, 2021). Molecular specific targeted therapy such as targeting DNA repair pathways, androgen receptor pathways and protein kinases

are treatment for TNBC. Monoclonal antibodies and small molecule inhibitors are the two compounds utilized for targeted therapies and the significant challenge associated with compounds is their ability to target only a certain subset of proteins or pathways. Targets for monoclonal antibodies are restricted to proteins on cellular membrane and extra cellular proteins and then small molecule inhibitors cannot target many proteins because of their extensive flat contact areas and absence of deep hydrophobic pockets that increases resistance to targeted therapy (Ngamcherdtrakul and Yantasee, 2019). Despite advances in treatment modalities, BC subtype TNBC which is aggressive and high metastatic possess challenges such as treatment resistance, disease relapse, and adverse effects persist, underscoring the need for innovative therapeutic approaches.

#### 1.4 COMBINATION THERAPY FOR TREATMENT OF BREAST CANCER

Even though significant advances in research have been made, BC has high incidence and mortality rates. Currently, various therapies are used to treat BC, including endocrine therapies, cytotoxic chemotherapies, and targeted therapies. While a wide range of treatments have been developed, the overall survival rate remains low, and some types of breast cancer, such as triple-negative breast cancer, are particularly difficult to treat. Over the last 50 years, BC treatment evolves from a single approach to more personalized treatments that focus on the characteristics of the tumor and the patient. In the last two to three decades, tremendous progress has been made in the development of targeted therapies drugs that are directed to a specific characteristic of a tumor, like a protein or a cellular process that is driving the tumor's growth (Hashemi *et al.*, 2021). Despite monotherapies (single agent) such as targeted therapy, hormone therapy, immunotherapy, or chemotherapy have shows a good efficiency for treatment of BC and many of these promising agents have been success with the use of monotherapies but some patients do not respond well, especially those with metastatic BC, largely because of their effectiveness sometimes weakens over time, and some patients develop resistance to treatment then tumor's ability to activate alternate growth-promoting pathways to overcome the therapy's effects. Even among patients who initially respond to monotherapy treatment, many eventually relapse so development of more effective and targeted treatments for BC is thus urgently needed (Correia *et al.*, 2021).

Scientists discover a new and promising idea is the use of combination therapy, where two or more agents are used for better therapeutic effectiveness in treatment of different diseases, such as infectious diseases and different types of cancer. Conversely, combination therapy, which combines two therapeutic treatments, has become a cornerstone of cancer therapy. Various types of combination therapy are currently being used in the clinic and in clinical trials (Bayat Mokhtari *et al.*, 2017). Several new combinational approaches have been developed. As this type of treatment becomes more widespread, technological advances are likely to lead to even more innovative approaches. Furthermore, supportive data such as nanotechnology, DNA sequencing technologies, and computational analysis can be used to optimize different combination approaches. Combination therapies can have significant advantages over monotherapies such as less toxicity, increased efficacy, and decreased dosage at an equal or increased level of efficacy and possibility of antagonizing drug resistance in cancer therapy (Wang and Minden, 2022).

### 1.5 TYROSINE KINASE INHIBITORS: A NEW HOPE IN CANCER TREATMENT

One promising approach to BC treatment involves the use of tyrosine kinase inhibitors (TKIs), which have shown promise in inhibiting tumor growth and angiogenesis in various cancers. In the human genome, ninety tyrosine kinases have been identified, including fifty-six receptor tyrosine kinases and thirty-two cellular tyrosine kinases. Tyrosine kinases lead to altering of the cell cycle, angiogenesis and lack of apoptosis in epithelial malignant cells (Paul and Mukhopadhyay, 2004). TKIs are a class of targeted therapies designed to perturb the cellular pathways that regulate malignant cell growth. TKI can be generally categorized into small molecules and macromolecules (e.g., monoclonal antibodies, polypeptides, antibody–drug conjugates, and nucleic acids). TKIs target signaling pathways involving the receptor tyrosine kinase and/or intracellular kinases that regulate cellular proliferation and tumor angiogenesis. The selectivity of binding of various TKIs to their targets influences the potency, mechanism of action, selectivity, and safety profile of these agents (Shyam Sunder *et al.*, 2023). TKIs play a crucial role in BC treatment by targeting specific signaling pathways involved in cancer progression. In BC, TKIs inhibit tyrosine kinases, such as epidermal growth factor receptors (EGFRs) and human epidermal growth factor

receptors (HERs), which are implicated in cell growth, survival, and metastasis. TKIs like lapatinib, neratinib, and pyrotinib have shown efficacy in HER2-positive breast cancer, either as single agents or in combination with other therapies (Yang *et al.*, 2020).

Axitinib (also known as AG-013736), is an oral, potent, and selective small molecule receptor tyrosine kinase inhibitor (TKI), exhibits potent activity against VEGF receptor (VEGFR)-1, VEGFR-2, and VEGFR-3, and, to a lesser extent, against platelet-derived growth factor receptor beta (PDGFR- $\beta$ ) and c-Kit. It has been approved for renal cell carcinoma by the US Food and Drug Administration (Rini *et al.*, 2005). Axitinib alone showed antitumor activity in multiple tumors containing thyroid cancer, non-small-cell lung cancer, melanoma, and so on (Fruehauf *et al.*, 2011). While axitinib has shown some promise in preclinical studies and clinical trials for BC treatment, further research is needed to determine its optimal use, potential synergies with other drugs, and its efficacy in specific subtypes or stages of BC. Monacolin-x, a secondary metabolite isolated from marine sponge *Clathria frondifera* associated fungi *Monascus sp.* NMK7 and showed antiproliferative/cytotoxic, antimigratory, apoptotic inducing ability on different breast cancer cells and angiogenesis inhibiting activity on HUVECs cells (Nagabhishek *et al.*, 2019; Nagabhishek and Madankumar, 2019). Both monacolin-x and axitinib have demonstrated anti-cancer effects individually. However, their potential synergy as a combination therapy against BC remains largely unexplored.

The purpose of this study is to investigate the efficacy of Axitinib, Monacolin-x, and their combination on MCF-7 and MDA-MB-231 cell line in-vitro and in-vivo.

## CHAPTER 2

### REVIEW OF LITERATURE

#### 2.1 COMBINATION THERAPY IN CANCER

Combination therapy is a treatment modality in which two or more therapeutic agents or different types of therapy such as immunotherapy, chemotherapy and radiotherapy are combined to enhance the treatment outcomes, overcome drug resistance and reduce side effects in various cancers, infectious diseases and chronic conditions. A study on combination of doxorubicin (DOX) and curcumin by (Zhang *et al.*, 2016b) demonstrated enhanced antitumor activity of the PEG-DOX-Cur NPs In HepG 2 and Hela cells then in xenograft mice with hepatic tumors. AZD6244 and MK2206 individually and combination on a large panel of lung cancer cell lines study by (Meng *et al.*, 2010) resulted in a synergistic effect on inhibition of lung cancer cell growth compared to the results of single drug treatment alone and the AZD6244-MK2206 combination therapy showed synergy in the suppression of A549 and H157 xenograft tumor growth. A novel model of combination that combines different CNS agents and antineoplastic drugs (5-fluorouracil (5-FU) and paclitaxel (PTX)) for colorectal and breast cancer therapy by (Duarte *et al.*, 2021) and in HT-29 cells, the combination of sertraline and thioridazine with 5-fluorouracil (5-FU) demonstrated the strongest synergism then in MCF-7 cells, the combination of fluoxetine, fluphenazine, and benztropine with paclitaxel (PTX) resulted in synergism. Combination of 5-FU and genistein on HT-29 colon cancer cells demonstrated abolished up-regulated state of COX-2 and prostaglandin secretion caused by 5-FU treatment in HT-29 colon cancer cells and the combination of 5-FU and genistein exert a novel chemotherapeutic effect in colon cancers (Hwang *et al.*, 2005). The combination of FTY720 with CDDP in a variety of ovarian cancer cell lines by (Zhang *et al.*, 2013) reported that the combination of FTY720 with CDDP yields an unexpected antagonistic effect towards the cytotoxicity of CDDP in a variety of ovarian cancer cell lines and the findings suggest that a combination of an autophagy regulator with the CDDP-based regime could effectively modulate its efficacy for the treatment of ovarian cancer

### **2.1.1 Combination Therapy in Breast Cancer**

A study to investigate the antitumor activity of arsenite (AsIII) and tetrandrine (Tetra) combination against MDA-MB-231 cells by (Yuan *et al.*, 2018) showed synergistic cytotoxic effects in vitro and in vivo along with S-phase arrest, apoptotic/necrotic cell death, and autophagic cell death. Combination of minocycline, an antibiotic that has previously displayed anti-cancer effects sabutoclax, a pan-Bcl-2 inhibitor by (Quinn *et al.*, 2015) discovered that acted synergistically on the intrinsic apoptotic pathway displayed selective toxicity and a reduction in tumour growth in vitro and in vivo on pancreatic ductal adenocarcinoma. Investigation of arsenite and tetrandrine in combination on human breast cancer cell line MCF-7 resulted in increased cytotoxicity, upregulation of FOXO3a expression, and subsequent upregulation of p21 and p27 expression, leading to G0/G1 phase arrest and exhibited a synergistic inhibitory effect on the expression of surviving (Yao *et al.*, 2017). An experiment by (Zhang *et al.*, 2016a) to evaluate the Synergistic combination of microtubule targeting anticancer fludellone with cytoprotective panaxytriol derived from panax ginseng against MX-1 cells in vitro indicated that the combination of FD and PXT exerted a synergistic effect when cell growth inhibition was greater than 45%, with CI values ranging from 0.836 to 0.609 for the fractional inhibition of  $F_a=0.50\sim0.90$ . The combination of trastuzumab and PARP inhibitor in HER2+ and HER2- breast cancer cells and xenograft model showed decreased cell viability and decreased inhibition in tumor growth in combination therapy, compared to vehicle and monotherapy-treated cohorts (Mansur *et al.*, 2023). Combination of PARP inhibitors, olaparib and rucaparib, with the anti-HER2 antibody trastuzumab on HER2+ breast cancer significantly decreased cell growth and enhanced anti-tumour effects of trastuzumab and combination treatment in the BT474 xenograft model resulted in enhanced growth inhibition, reduced tumour cell proliferation, and increased DNA damage and apoptosis (Garcia-Parra *et al.*, 2014).

## **2.2 TYROSINE KINASE INHIBITORS IN BREAST CANCER**

Tyrosine kinase inhibitors (TKIs) are a class of targeted therapy drugs that interfere with specific signaling pathways involved in the tumor growth and spread of cancer cells. These inhibitors work by blocking the activity of tyrosine kinases, enzymes that play a key role in cell signaling and regulation of cell growth. By

inhibiting these kinases, TKIs can help slow down or stop the growth of cancer cells and shown effectiveness in treating various cancers, including breast cancer. Combined inhibition of EGFR and PARP by lapatinib and ABT-888 achieved synthetic lethality both in vitro and in vivo and then induces DNA repair deficit and activates apoptosis in TNBC cells (Nowsheen *et al.*, 2012). To evaluate the antitumor activity and pharmacodynamic/biologic effect of gefitinib in advanced breast cancer (Baselga *et al.*, 2005) conducted a study that demonstrates a good correlation between the degree of inhibition of EGFR in skin and in breast tumor. To evaluate the safety and efficacy of metronomic chemotherapy in combination with bevacizumab and erlotinib in patients with HER2-negative (HER2(-)) metastatic breast cancer (Montagna *et al.*, 2012) conducted a study and it was found to be effective in combination with bevacizumab and erlotinib against HER2(-), estrogen receptor (ER)- and progesterone receptor (PR)-poor advanced breast cancer. Combination of letrozole plus lapatinib in (HER)-2(+) metastatic breast cancer reduced the risk of disease progression compared to letrozole alone, leading to a longer progression-free survival (PFS) time than with letrozole alone in women with MBC who coexpress HR and HER-2 and Currently, lapatinib is approved by Food and Drug Administration (FDA) for BC treatment in combination with letrozole (Schwartzberg *et al.*, 2010). The combination of gefitinib and taxane on the breast cancer cell lines MDA-MB-231 and MCF7/ADR cells showed a strong synergistic effect on MCF7/ADR cells and additive-antagonistic effect on MDA-MB-231 cells then similarly, the combination treatment showed a significantly increased tumor inhibitory effect on MCF7/ADR xenografts, but not on MDA-MB-231 xenografts (Takabatake *et al.*, 2007).

### 2.3 ROLE OF AXITINIB IN CANCER

Axitinib is a potent and highly selective tyrosine kinase inhibitor that targets vascular endothelial growth factor receptor (VEGFR) and is used in the treatment of advanced renal cell carcinoma (RCC), a type of kidney cancer. It works by blocking enzymes known as tyrosine kinases found in VEGF receptors on cancer cells, thereby inhibiting cancer cell growth and spread by cutting off the blood supply to tumors. To investigate the effect of axitinib on tumor growth and on the composition of tumor-infiltrating immune cells in subcutaneous and intracranial mouse melanoma models (Du Four *et al.*, 2015) conducted a study. In which,

axitinib significantly inhibits tumor growth and improves survival in both subcutaneous and intracranial melanoma models. Axitinib treatment results in a significant increase in the number of CD3+CD8+ T cells and CD11b+ cells in the spleen and tumor of tumor-bearing mice then the impact of axitinib on tumor growth and survival is most likely not restricted to direct anti-angiogenic effects but also involves important effects on tumor immunity. The antitumor activity of axitinib alone or combined with chemotherapeutic drugs 5-fluorouracil (5-FU) or cisplatin against human gastric cancer cells in vitro and in vivo showed synergistic inhibitory effect as combination compared to axitinib or 5-FU alone. However, the highest inhibitory effect was found between axitinib and cisplatin (*He et al.*, 2014). To evaluate the efficacy of Avelumab plus Axitinib versus Sunitinib for Advanced Renal-Cell Carcinoma (*Motzer et al.*, 2019) conducted a study. The combination of avelumab, and axitinib has demonstrated improved progression-free survival (PFS) and higher objective response rates (ORR) in patients with treatment-naive advanced RCC and compared avelumab plus axitinib to sunitinib and found that the combination therapy resulted in a tolerable safety profile and encouraging antitumor activity. Avelumab plus axitinib has shown promising results in the treatment of advanced renal-cell carcinoma (RCC) compared to sunitinib. A study by (*Mongiardi et al.*, 2019) reported that axitinib in second line treatment for advanced renal carcinoma promotes senescence of human endothelial cells in vitro and axitinib exposure triggers senescence in endothelial cells through the accumulation of reactive oxygen species (ROS) and activation of the Ataxia Telangiectasia Mutated (ATM) kinase. Combination therapy with axitinib and crizotinib in mice models of castration resistant prostate cancer remarkably inhibited the tibial remodeling by VCaP-Luc cells which resulted in a significant reduction of both osteoblastic and osteolytic lesions in mouse models (*Eswaraka et al.*, 2014). Phase I study of axitinib combined with paclitaxel, docetaxel or capecitabine in patients with advanced solid tumours by (*Martin et al.*, 2012) reported that axitinib combined with paclitaxel or capecitabine was well tolerated; no additive increase in toxicities was observed across multiple tumour types. A study conducted by (*Dicitore et al.*, 2023) to evaluate the antitumor activity of axitinib on human lung TC (NCI-H727, UMC-11, NCI-H835) and AC (NCI-H720) cell lines by collecting both in vitro and in vivo. Axitinib demonstrated relevant antitumor activity in human LC cells, with pronounced effects observed in UMC-11

and NCI-H720 and significantly hindered tumor induced-angiogenesis in Tg(fli1a:EGFP)<sup>y1</sup> zebrafish embryos implanted with all LC cell lines and also reduced the invasiveness of NCI-H720 cells, as well as the secretion of several proangiogenic factors. In canine mammary gland cell lines CIPp and CIPm the antitumor activity of axitinib significantly decreased cell viability at 24, 48, and 72 h, and reduced migration also the apoptosis rate was significantly increased, and the G2/M phase ratio showed a significant increase. Additionally, there was no significant change in the viability of canine PBMCs treated with LPS and ConA (Lee *et al.*, 2024). A preclinical assessment of the VEGFR inhibitor axitinib as a therapeutic agent for epithelial ovarian cancer cells (A2780, HeyA8, RMG1, and HeyA8-MDR) and in vivo experiments in xenografts resulted in significant inhibition of cell survival and migration, and increased apoptosis in EOC cells. In in vivo experiments, axitinib significantly decreased tumor weight in xenograft models of drug-sensitive (A2780), and clear cell carcinoma (RMG1) and PDX models for platinum sensitive EOC compared to control, but was not effective in drug-resistant cell line (HeyA8-MDR) or heavily pretreated refractory PDX model (Paik *et al.*, 2020).

### **2.3.1 Role of Axitinib in Breast Cancer**

To evaluate the effect of dopamine (DA) improving AX's efficacy on MCF-7/ADR breast cancer in vitro and in vivo (Ma *et al.*, 2019) developed a pharmacokinetic-pharmacodynamic (PK-PD) model describing the in vivo experimental data and characterizing the interaction of effect between AX and DA. The results showed that AX up-regulated the expression of breast CSC (BCSC) markers (CD44<sup>+</sup>/CD24<sup>-/low</sup>) in vivo, and DA significantly synergized the inhibitory effect on tumor growth by deducting the BCSC frequency and the outcome of AX and DA combination therapy in MCF-7/ADR breast cancer provided further insight of co-administering DA in the treatment of the possible CSC-causing AX-resisting breast cancer. A study by (Hu-Lowe *et al.*, 2008) on nonclinical antiangiogenesis and antitumor activities of axitinib (AG-013736), an oral, potent, and selective inhibitor of vascular endothelial growth factor receptor tyrosine kinases 1, 2, 3. The results showed that xitinib inhibits cellular autophosphorylation of VEGF receptors (VEGFR) with picomolar IC50 values. Counterscreening across multiple kinase and protein panels shows it is selective for VEGFRs and axitinib inhibits platelet-

derived growth factor receptors and KIT with nanomolar in vitro potencies, based on pharmacokinetic/pharmacodynamic analysis, axitinib acts primarily as a VEGFR tyrosine kinase inhibitor at the current clinical exposure. A randomized, placebo-controlled, double-blind, phase II study of axitinib plus docetaxel versus docetaxel plus placebo in patients with metastatic breast cancer by (Rugo *et al.*, 2011) did not improve Time to Progression in first-line MBC treatment and suggested that the addition of axitinib to docetaxel Combination therapy may be more effective in patients previously exposed to adjuvant chemotherapy. A study on axitinib transport using Madin-Darby canine kidney II cells overexpressing human ABCB1 or ABCG2 or murine Abcg2 by (Poller *et al.*, 2011) and the results revealed that ABCB1 strongly restricts axitinib brain accumulation and compensates for the loss of ABCG2 at the blood-brain barrier, while ABCG2 only partially takes over ABCB1-mediated axitinib efflux.

## 2.4 ROLE OF MONACOLIN-X IN CANCER

Monacolin, also known as lovastatin, is a natural statin drug that is commonly used to lower cholesterol levels in individuals at risk of cardiovascular diseases. While its primary mode of action is to inhibit the enzyme HMG-CoA reductase, which is involved in cholesterol synthesis. A study reported that *Monascus ruber* M82121 a mutant strain treated with UV irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, produces monacolin-x along with monacolins J and L (Endo *et al.*, 1985). A novel apoptosis-inducing metabolite from marine sponge symbiont *Monascus* sp. NMK7 associated with the marine sponge *Clathria frondifera* was isolated by (Nagabhishek and Madankumar, 2019) and the isolated metabolite with molecular weight of 418.5 Da and molecular formula C<sub>24</sub>H<sub>34</sub>O<sub>6</sub>, indicating the presence of monacolin-x then was found to have a promising antiproliferative property breast cancer cells. A study to investigate the angiogenesis suppression via down regulation of VEGFR2 signaling by monacolin-x a marine fungal secondary metabolite. In which, monacolin x suppresses in vitro angiogenic characteristics such as proliferation, migration, adhesion, invasion and tube formation of HUVECs cell when stimulated by VEGF, at a non-toxic concentration. Further, monacolin-x inhibited in vivo angiogenesis in CAM assay, vascular sprouting in aortic ring, decreased ISV and SIV length and diameter in Tg (Kdr:EGFP)/ko1 zebrafish embryos. In silico studies also revealed monacolin-x

binding ability to crucial amino acid Cys 919 in the active site of VEGFR2 suggesting it to be a potent VEGFR2 inhibitor (Nagabhishek *et al.*, 2019).

## 2.5 ROLE OF TNF-A IN BREAST CANCER

Tumor necrosis factor alpha (TNF- $\alpha$ ) is a pro inflammatory cytokine and a classical pro-angiogenic factors-induced signaling pathway, which is prominent in the tumor microenvironment under hypoxic conditions that plays a complex role in cancer including BC (Ansari *et al.*, 2022). TNF- $\alpha$  has been associated with promoting the growth and proliferation of breast cancer cells, including TNBC. It can stimulate signals for activation, differentiation, survival, or cell death, highlighting its diverse effects on tumor behavior (Cai *et al.*, 2017). TNF- $\alpha$  mediating its action through cell-surface receptors and It has been implicated in promoting angiogenesis through various mechanisms (Bigda and Okrój, 2002). TNF- $\alpha$  primes endothelial cell for angiogenic sprouting by inducing proangiogenic genes like VEGFR2, PDGFB, and jagged-1. It also modulates VEGFR2 signaling and promotes endothelial cell migration and the effects of TNF- $\alpha$  on angiogenesis are multifaceted and can vary based on the duration of exposure. Continuous TNF stimulation may suppress angiogenesis, while a pulsed exposure can enhance vascularization by priming endothelial cells for sprouting (Sainson *et al.*, 2008).

## 2.6 ROLE OF IL-6 IN BREAST CANCER

Interleukin-6 (IL-6) is a pleiotropic pro inflammatory cytokine and a classical pro-angiogenic factors-induced signaling pathway, which is prominent in the tumor microenvironment under hypoxic conditions with significant implications in breast cancer, particularly TNBC (Ansari *et al.*, 2022). IL-6 expression induces an Epithelial-to-Mesenchymal Transition (EMT) phenotype in breast cancer cells, promoting tumor growth, invasion, migration, and the enrichment of Cancer Stem Cells (CSCs) and mediates metastasis (Manore *et al.*, 2022). IL-6 signaling pathway is implicated in promoting angiogenesis by stimulating the expression of factors like VEGF, IL-8 and FGF-2 that are crucial for blood vessel formation and this involvement underscores the significance of IL-6 in regulating vascularization processes within the tumor microenvironment (Masjedi *et al.*, 2018).

## 2.7 ROLE OF IL-1B IN BREAST CANCER

Interleukin-1 beta (IL-1 $\beta$ ), a pro-inflammatory cytokine that drives the recruitment of Tumor-Associated Macrophages (TAMs), immunosuppression and has been implicated in promoting tumor growth, invasion, metastases, stemness, and Epithelial-to-Mesenchymal Transition (EMT) in breast cancer (Wilson *et al.*, 2023). IL-1 $\beta$  has been characterized as a principal effector molecule in initiating angiogenesis through matrix metalloproteinase (MMP)-induced sequestration of extracellular matrix components and subsequent ligation to VEGFR2 on endothelial cells. IL-1 $\beta$  is a key player in promoting inflammatory and angiogenic responses within the tumor microenvironment and IL-1 $\beta$  can regulate angiogenesis similarly to Vascular Endothelial Growth Factor (VEGF) in endothelial cells (Nisar *et al.*, 2021).

## 2.8 ROLE OF NF-KB IN BREAST CANCER

Nuclear factor-kappa B (NF- $\kappa$ B), a proinflammatory transcription factor, associated with aggressive tumor biology, poor prognosis, and chemotherapy resistance. It is detected in the nucleus of cancer cells in various tumors, including breast cancer and it is constitutively activated particularly in aggressive estrogen receptor (ER)-negative and TNBC subtypes (Wang *et al.*, 2015). NF- $\kappa$ B activation is linked to promoting neovascularization, epithelial-mesenchymal transition (EMT), increasing cancer cell stemness and leading to resistance to chemotherapy, radiotherapy, and endocrine therapy. NF- $\kappa$ B activation promotes angiogenic neovascularization in breast cancer, contributing to the establishment and maintenance of cancer-related angiogenesis (Shostak and Chariot, 2011). The upregulation of NF- $\kappa$ B signaling, either alone or in conjunction with other pathways, influences various processes like EMT and Inhibition of NF- $\kappa$ B could potentially disrupt these angiogenic responses and hinder tumor progression (Pavitra *et al.*, 2023).

## **CHAPTER 3**

### **AIM & OBJECTIVE**

#### **AIM**

To investigate the therapeutic efficiency of monacolin-x and axitinib as a combination for treatment against human breast cancer cell line.

#### **OBJECTIVE**

- ✚ To explore the potential of monacolin-x and axitinib in inhibition of breast cancer cells as a combination therapy.
- ✚ To evaluate the combined effects of monacolin-x and axitinib as a combination therapy.
- ✚ To determine the optimal dosage range and treatment regimen for combination therapy of monacolin-x and axitinib.
- ✚ To explore the underlying molecular mechanism of action of monacolin-x and axitinib as a combination therapy.

## CHAPTER 4

### MATERIALS AND METHODS

#### 4.1 CHEMICALS AND REAGENTS

Axitinib (Sigma, #PZ0193). Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide), Dimethyl sulfoxide (DMSO), Propidium iodide (PI), Acridine Orange (AO) and Ethidium bromide (EB), DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate), DPPH(2,2-diphenyl-1-picrylhydrazyl) were purchased from Hi-media (California, USA). All other required reagents were bought from Sigma Aldrich (USA) and were of analytical grade.

#### 4.2 IN VITRO STUDIES

##### **4.2.1 Cell line and Culture**

MCF-7 and MDA-MB-231; breast cancer cell lines were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in T25 culture flasks containing DMEM media and supplemented with 10% FBS containing 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and amphotericin-B (1x solution). The Cells were maintained in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. The cells were sub-cultured Upon reaching 70%-80% confluency, the spent media was removed and cells were washed with 2ml of sterile PBS (1x solution) twice to remove the debris and contaminants and then 2ml of trypsin-EDTA solution was added to detach the adherent cells from the surface of T25 flask and incubated at 37°C in 5% CO<sub>2</sub> for 3 min to ensure the cells are detached, to inactivate the trypsin 2ml of DMEM media supplemented with 10% FBS was added to the T25 flask and transferred it to the 15ml centrifuge tube and then centrifuged at 900 rpm for 3 min. After centrifugation, the supernatant was discarded and pellet was dissolved in DMEM media supplemented with 10% FBS and then the cell density were counted using haemocytometer for further assay.

**TABLE 4.1 List of cell lines**

| Type of Cell line                   | Cell line  | Medium used       | Source of cell line                                  |
|-------------------------------------|------------|-------------------|------------------------------------------------------|
| Control Breast cancer cells         | MCF7       | DMEM with 10% FBS | National Centre for Cell Science (NCCS), Pune, India |
| Triple negative Breast cancer cells | MDA-MB-231 | DMEM with 10% FBS | National Centre for Cell Science (NCCS), Pune, India |

#### **4.2.2 Preparation of the Sample**

The chemical Axitinib (AG-013736) (N-methyl-2-[3-(E)-2-pyridin-2-yl-vinyl-1H-indazol-6-yl-sulfanyl] benzamide) was commercially brought from sigma Aldrich (USA) containing 5mg/ml concentration in the powdered form, the molecular weight of Axitinib ( $C_{22}H_{18}N_4OS$ ) is 386.469 g/mol .For *In-vitro* and *In-vivo* experiments, axitinib was dissolved in 10mM/ml in 1% DMSO and diluted as needed. The chemical Monacolin-x ( $C_{24}H_{34}O_6$ ) with molecular weight 418.5 g/mol was isolated from marine sponge *Clathria frondifera* associated fungi *Monascus* sp. NMK7. For *in-vitro* and *In-vivo* experiments, monacolin-x was dissolved in 40mg/ml in 1% DMSO and diluted as needed.

#### **4.2.3 Evaluation of growth inhibition and cytotoxicity of Axitinib and Monacolin-x combination**

The growth inhibition and cytotoxicity of the drugs axitinib and monacolin-x was analysed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay on MCF-7 and MDA-MB-231 cell lines. MTT assay relies on the ability of viable cells after treatment to reduce the yellow tetrazolium salt MTT compound into a purple insoluble formazan crystals by NAD(P)H-dependent cellular oxidoreductase mitochondrial enzymes in viable cells. MCF-7 and MDA-MB-231 cells about 70%-80% confluent cells were washed with 2ml of sterile PBS

(1x solution) and trypsinized with 2ml of trypsin-EDTA solution to detach the adherent cells for sub-culture and the cell counting was done using haemocytometer then seeded into a 96-well plate at a density of  $1 \times 10^4$  cells/well with 10% DMEM media supplemented with FBS and incubated for 24hrs at 37°C in 5% CO<sub>2</sub> humidified atmosphere. After 24hrs incubation, the spent media in 96-well plate was removed and washed with 10µl of sterile PBS (1x solution) to remove the debris and contaminants. after washing, PBS was removed and 10% DMEM media supplemented with FBS was added to the 96-well plate and were treated with different concentrations of the drugs axitinib alone (5µM, 10µM, 15µM, 20µM, 25µM, 30µM, 50µM), monacolin-x alone (10µM, 20µM, 30µM, 40µM, 50µM, 75µM, 100µM) and axitinib+monacolin-x (5µM+10µM, 10µM+20µM, 15µM+30µM, 20µM+40µM, 25µM+50µM, 30µM+75µM, 50µM+100µM) in combination. After exposed to treatment with different concentrations of the drugs axitinb and monacolin-x, the 96-well plate was incubated for 24hrs at 37°C in 5% CO<sub>2</sub> humidified atmosphere. Following 24hrs incubation, 50µl of MTT (5mg/ml of stock in PBS) is added to the 96-well plate and incubated for 4hr under dark conditions at 37°C in 5% CO<sub>2</sub> humidified atmosphere. After incubating for the required amount of time, the media was removed and 100 µL of DMSO was added to the 96-well plate to dissolve the insoluble formazan crystals and then incubated for 20 min at room temperature. Finally the MTT reduction optical density was quantified by measuring the absorbance at 570nm using multiwell microplate reader (Agilent Biotek, California, U.S). The experiment is performed in triplicates. The IC<sub>50</sub> value of the drugs axitinib and monacolin-x as alone and combination was calculated using the formula.

$$\% \text{ cell viability} = \frac{(Abs \text{ control} - Abs \text{ sample})}{Abs \text{ control}} \times 100$$

$$\% \text{ cell inhibition} = 100 - \left( \frac{(Abs \text{ control} - Abs \text{ sample})}{Abs \text{ control}} \right) \times 100$$

#### 4.2.3.1 Evaluation of Axitinib and Monacolin-x combination effects

The combination effect of axitinib and monacolin-x on MCF-7 and MDA-MB-231 cells were evaluated by the combination index (CI), the IC<sub>50</sub> values of axitinib and monacolin-x as individual and as combination were obtained performing MTT assay and then fraction affected (Fa) values of axitinib and monacolin-x as alone and as combination was calculated to assess the fraction of cells affected and to evaluate the effectiveness of drugs as individual and as combination at a given concentration. The combination effects of the drugs axitinib and monacolin-x were defined as CI<1 indicates synergistic effect, CI=1 indicates additive effect and CI>1 indicates antagonistic effect (Takabatake et al., 2007).

$$\text{Fraction affected (Fa)} = 1 - \left( \frac{\text{Abs of sample}}{\text{Abs of control}} \right)$$

$$\text{Combination Index (CI)} = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2} + \frac{(D)1(D)2}{(Dx)1(Dx)2}$$

where (Dx) 1 is the dose of Drug 1 required to produce an X% effect alone, (D) 1 is the dose of Drug 1 required to produce the same X% effect in combination with Drug 2, (Dx) 2 is the dose of Drug 2 required produce an X% effect alone, and (D) 2 is the dose of Drug 2 required to produce the same X% effect in combination with Drug 1.

#### 4.2.4 Detection of Cellular Apoptosis

Apoptosis is a programmed cell death, an important physiological process for the maintenance of tissue homeostasis and plays a pivotal role in the pathogenesis of various diseases. Apoptosis occurs without eliciting a local inflammatory response, in the case of cancer therapy, one of the most important aspects is to induce apoptosis and kill cancer cells and the endothelial cells. The apoptosis induction of the cell can be visualized by two different staining methods.

##### 4.2.4.1 Propidium iodide (PI) staining

The apoptotic effect of axitinib and monacolin-x as alone and as a combination was analysed by morphologically assessing cells using nuclear dye

Propidium iodide staining. PI is a nucleic acid fluorescent dye which is impermeable and was done to distinguish between apoptotic cells from normal cells. It is used to visualize chromatin condensation and fragmentation as it stains the condensed nuclei of apoptotic cells and can be visualized in fluorescence microscope. MDA-MB-231 cells about 70%-80% confluency were trypsinized for sub-culturing and seeded into a 6-well plate at a density of  $2 \times 10^4$  cells/well with 10% DMEM media supplemented with FBS and incubated for 24 hrs at 37°C in 5% CO<sub>2</sub> humidified atmosphere. After incubation, cells were treated with the IC50 concentration of axitinib, monacolin-x as individual and axitinib+monacolin-x combination with one untreated well as control then incubated for 24 hr at 37°C in 5% CO<sub>2</sub> humidified atmosphere. PI stock was prepared by dissolving PI (1 mg/mL) in PBS (1X solution) and further diluted to obtain working solution. After 24 hrs treatment, the spent media was discarded, washed with sterile PBS (1X solution) twice. Then the cells were stained with 100µl of PI (10 µg/mL) and incubated at 37°C for 15mins in dark conditions. The fluorescent images were viewed under EVOS fl AMG inverted fluorescence microscope. The formation of apoptotic bodies, nuclear condensation were visualized as the result of this apoptotic detection in cells.

#### *4.2.4.2 Acridine Orange (AO) / Ethidium Bromide (EB) staining*

The apoptotic inducing activity of axitinib and monacolin-x as alone and as a combination was assessed by performing AO and EB dual staining for visualization of nuclear modification. AO is a vital dye that stains both live cells and dead cells whereas EB stains only dead cells that have lost membrane integrity. AO and EB dual staining method can accurately distinguish the cells in different stages of apoptosis as early apoptotic cells, late apoptotic cells and necrotic cells. Early apoptotic cells stain green and characterized by yellowish-green fluorescence contain bright dots in the nuclei. Late apoptotic cells stain green and also incorporate EB and show condensed and often fragmented nuclei that appears orange in color as a mixture of both green and red because late apoptotic cells take both AO and EB. Necrotic cells stain in red and viable cells stain in green. MDA-MB-231 cells about 70%-80% confluency were trypsinized for sub-culturing and seeded into a 6-well plate at a density of  $2 \times 10^4$  cells/well with 10% DMEM media supplemented with FBS and incubated for 24 hrs at 37°C in 5%

CO<sub>2</sub> humidified atmosphere. Later, cells were treated with IC<sub>50</sub> concentration of axitinib, monacolin-x as individual and axitinib+monacolin-x combination with one untreated well as control then incubated for 24 hrs at 37°C in 5% CO<sub>2</sub> humidified atmosphere. Following treatment, the spent media was discarded and washed with sterile PBS (1X solution) twice. Then, 10 µl of (1 mg/mL) each AO and EB is added in the ratio of 1:1 in the 6-well plate and incubated for 20 mins. Apoptotic cells morphology like membrane blebbing and apoptotic bodies formation was viewed and photographed under the EVOS fl AMG inverted fluorescent microscope.

#### ***4.2.5 Measurement of intracellular reactive oxygen species (ROS)***

Intracellular ROS was measured using a cell-permeable fluorescent dye DCFH-DA (2', 7'-dichlorodihydrofluorescein diacetate). DCFH-DA diffuses through the cell membrane and is hydrolyzed by an intracellular esterase to the non-fluorescent dichlorofluorescein (DCF), which is rapidly converted into the extremely fluorescent compound dichlorofluorescein in the existence of peroxidase enzyme by intracellular ROS. Therefore, the fluorescent amount is directly related to the quantity of ROS produced from the cells (Rastogi et al., 2010). MDA-MB-231 cells about 70%-80% confluency were trypsinized for sub-culturing and seeded into a 6-well plate at a density of  $2 \times 10^4$  cells/well with 10% DMEM media supplemented with FBS and incubated for 24 hrs at 37°C in 5% CO<sub>2</sub> humidified atmosphere. After 24 hrs incubation, the spent media was removed and cells were washed with sterile PBS (1x solution) once to remove the debris and contaminants. After washing, PBS was removed and 10% DMEM media supplemented with FBS was added to the 6-well plate then treated with IC<sub>50</sub> concentrations of the axitinib, monacolin-x as individual and axitinib+monacolin-x combination with one untreated well as control then incubated for 24 hrs at 37°C in 5% CO<sub>2</sub> humidified atmosphere. After 24 hrs treatment, DCFH-DA dissolved in DMSO was added to the 6-well plate at a concentration of 10 µM and incubated in dark conditions at 37°C in 5% CO<sub>2</sub> incubator for 30 min. After staining, the 6-well plate was washed with sterile PBS (1x solution) and resuspended in sterile PBS (1x solution) and then images were taken qualitatively by EVOS fl AMG inverted fluorescent microscope and quantitatively measured the ROS level at 488 nm Excitation and

525 nm Emission by multiwell microplate reader (Nagabhishek and Madankumar, 2019).

#### **4.2.6 Evaluation of Antioxidant activity of Axitinib and Monacolin-x combination**

To evaluate the antioxidant effect, the free radical scavenging activities of axitinib and monacolin-x was determined by DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging method. DPPH is a stable free radical compound that accepts an electron or hydrogen atom to become a stable diamagnetic molecule. When an antioxidant compound is added to a DPPH solution, it can donate an electron or hydrogen atom to the DPPH radical, neutralizing its free radical nature. DPPH assay is based on the colour change of the DPPH radical from purple to yellow as it is reduced by an antioxidant compound. The degree of colour change is directly proportional to the antioxidant activity of the compound being tested. DPPH stock solution (1 mM) was prepared by dissolving 19.715 mg of DPPH in ethanol for 50ml and the working solution of DPPH (150 µM) was prepared by dissolving 7.5ml from the stock in 42.5ml of ethanol and stored in dark. axitinib alone (5µM,10µM,15µM,20µM,25µM,30µM,50µM), monacolin-x alone (10µM,20µM,30µM,40µM,50µM,75µM,100µM) and axitinib+monacolin-x (5µM+10µM,10µM+20µM,15µM+30µM,20µM+40µM,25µM+50µM,30µM+75µM,50 µM+100µM) in combination was added in triplicates in a 96-well plate with standard control ascorbic acid (5 µl, 10 µl, 15 µl, 20 µl, 25 µl, 30 µl, 50 µl). Then 100 µl of DPPH solution was added and incubated for 30 minutes at room temperature in the dark. After incubating for appropriate period of time, the absorbance was measured at 517 nm by multiwell microplate reader and the percentage of inhibition of DPPH radical was calculated using the formula.

$$\% inhibition = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100$$

#### **4.2.7 Total RNA Isolation**

MDA-MB-231 cells about 70%-80% confluency were trypsinized for sub-culturing and seeded into a 6-well plate at a density of  $1 \times 10^6$  cells/well with 10% DMEM media supplemented with FBS and incubated for 24 hrs at 37°C in 5%

CO<sub>2</sub> humidified atmosphere. Following incubation, axitinib and monacolin-x was administered at IC<sub>50</sub> concentrations as individual treatment and as combination treatment then incubated for 24 hr in 5% CO<sub>2</sub> incubator. After incubation, cells were washed with ice cold sterile PBS (1X solution) and removed. To isolate the total RNA from control and treated cells, 1ml of TRIzol reagent was added and the cells are scrapped off from the surface of 6-well plate using cell scrapper then TRIzol/cell lysate was transferred into a 1.5ml eppendorf tube and the cell lyaste was incubated at room temperature for 5 minutes. To the cell lysate, 200 µl of chloroform was added then vortexed the cell lysate vigorously for 15 seconds and incubated at room temperature for 5 minutes. The cell lysate was centrifuged at 10,000 rpm for 5 minutes at 4°C. After centrifugation, the mixture was separated into three phases; a lower red-phenol chloroform organic phase, interphase containing precipated DNA and a colorless upper aqueous phase contains RNA. The upper aqueous phase contains RNA was pipetted out without disturbing the interphase and transferred into another 1.5ml eppendorf tube. To precipitate the RNA, 500 µl of isopropanol was added to the aqueous phase and leaved at room temperature for 5 minutes. Then the sample was centrifuged at 14,000 rpm for 20 minutes at 4°C. After centrifugation, a pellet was barely visible at the bottom of the tube and then the supernatant was discarded without disturbing the pellet. The RNA pellet was washed with 1ml of 75% ethanol then the pellet was dissolved by vortexing and recentrifuged at 8000 rpm for 5 minutes at 4°C. After centrifugation, the ethanol was poured off and let the pellet to air dry for 10 minutes. The dried pellet was resuspended by adding 50 µl of sterile nuclease free water and vortexed thoroughly. The isolated RNA content was quantified at 260 nm and 280 nm using a microplate reader to determine sample concentration, purity of RNA and the amount of RNA to be taken per µl. The RNA sample was stored at -80°C until further use.

#### **4.2.8 cDNA Conversion**

100 ng of total RNA was converted to cDNA, using iScript™ cDNA Synthesis Kit according to the manufacturer's protocol in a two-step procedure. 15 µL of total RNA was mixed with 4 µL of the master mix and 1 µL of Reverse Transcriptase (RT) enzyme then made upto 20 µL according to the protocol. The

tubes were incubated in a thermal cycler at 25°C for 5 min for priming, 46°C for activating RT enzyme, then at 95°C for 1 min to inactivate reverse transcriptase and were held at 4°C then the tubes were stored at -20°C for further use.

#### ***4.2.9 Designing Primers for quantitative Real-Time-Polymerase Chain Reaction***

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

**TABLE 4.2 List of Primers used for qRT-PCR analysis**

| S.NO | TARGET GENE | FORWARD PRIMER                           | REVERSE PRIMER                            | AMPLICON LENGTH (bp) | Ta ( °C) |
|------|-------------|------------------------------------------|-------------------------------------------|----------------------|----------|
| 1    | NF-κB       | 5'-<br>CGAATGGACA<br>AGACAGCAG-<br>3'    | 5'-<br>CCGGAACACA<br>ATGGCATAC-<br>3'     | 177                  | 53.7     |
| 2    | IL 6        | 5'-<br>ATAACCACCC<br>CTGACCCCAA<br>C-3'  | 5'-<br>ATACCTCAAA<br>CTCCAAAAGA<br>CC-3'  | 149                  | 55.1     |
| 3    | TNF α       | 5'-<br>AACCATTCTC<br>CTTCTCCCC-3'        | 5'-<br>TTTCACCTCC<br>ATCCATCCTC-<br>3'    | 194                  | 54.3     |
| 4    | IL 1β       | 5'-<br>CTCTCACCTC<br>TCCTACTCAC-<br>3'   | 5'-<br>ACACTGCTAC<br>TTCTTGCCC-<br>3'     | 186                  | 51.5     |
| 5    | GAPDH       | 5'-<br>GAAGGTCGGA<br>GTCAACGGAT<br>TT-3' | 5'-<br>CTGGAAGATG<br>GTGATGGGAT<br>TTC-3' | 183                  | 56.1     |

#### ***4.2.10 Gene Expression analysis using Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)***

qRT-PCR was performed with gene-specific primers for four genes (NF-κB, IL-6, TNF α, IL-1β) using SensiFAST™ SYBR® Lo-ROX Kit (Bioline, UK) according to the manufacturer's protocol in duplicates. 5μL of SYBR Green master mix, 1μL of forward primer (3μM), 1μL of reverse primer (3μM), and 3μL of cDNA template (50 ng) was added and the volume was made to 10μL with nuclease-free

water. PCR amplification was performed with the Mastercycler Nexus Gradient Thermal Cycler (Eppendorf, Germany). The thermal-cycling conditions included 95°C for 2 min (polymerase activation), 95°C for 5 sec, 60 to 65°C for 10 sec, 72°C for 15 sec for 40 cycles. The program was set up to calculate Cycle threshold value (Ct value) for 40 cycles. The relative expression levels of the mRNA of the target genes were normalized using the GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) internal standard gene as control. The relative expression levels between target and control were measured with the Relative quantification ( $2^{-\Delta\Delta Ct}$ ) method. In this method, the relative gene expression is assumed to be 1 for the reference sample. It assumes 100% efficiency and uniform PCR amplification across all the samples. The results were reported as expression fold change when compared to axitinib, monacolin-x and control. Fold change >1.0 was considered as upregulation and fold change < 1.0 was considered as downregulation in the gene expression.

#### 4.3 IN VIVO STUDIES

##### **4.3.1 Maintenance and Spawning of Zebrafish (*Danio Rerio*)**

Adult zebrafish were purchased and maintained at 28.5°C with a photoperiod of 14hr light and 10hr dark. The fishes were fed twice a day with dry shrimp flakes and water was changed periodically. The adult acclimatized zebrafish female and male at 1:2 ratios was used for spawning. In order to spawn, zebrafishes were confined in a plastic mesh to prevent cannibalism of the released eggs and covered with black plastic for 12 hours to provide dark condition. Following incubation, eggs were exposed to light, and spawning was triggered once the light was turned on and was completed within 30 minutes. After fertilization, embryos at segmentation phase were collected, rinsed, and observed under a stereomicroscope. Unfertilized embryos were removed prior to its use in further experiments (Truong *et al.*, 2011).

##### **4.3.2 Zebrafish Embryo toxicity and teratogenicity Assay**

The zebrafish embryo toxicity assay was performed under the guidelines of OCED (Organisation for Economic Co-operation and Development). Though

axitinib and monacolin-x was accounted for anti-cancer activity, it is essential to check their toxic nature. Embryotoxicity and teratogenicity is an in-vivo toxicity test using zebrafish embryos to evaluate the toxic nature of the drugs axitinib and monacolin-x as individual and as combination. Ten embryos at segmentation phase were collected and placed in a 12-well plate containing E3 medium (5mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, 0.33 mM MgSO<sub>4</sub>, and 0.1% Methylene Blue) which is changed periodically. Axitinib and monacolin-x was prepared by diluting the IC<sub>50</sub> concentration of axitinib and monacolin-x in 1ml E3 medium for zebrafish embryo treatment. The embryos were treated with various concentrations of axitinib (10µg/mL, 20µg/mL and 40µg/mL), monacolin-x (5µg/mL, 10µg/mL and 20µg/mL) and axitinib+monacolin-x (5µg/mL+20µg/mL, 10µg/mL+10µg/mL and 20µg/mL+5µg/mL) with one untreated well as control in a 12-well plate. The 12-well plate was incubated at 26°C with a photoperiod of 14hr light and 10hr dark. The teratogenic activities of treated embryos were viewed using a stereomicroscope after 24h, 48h, 72h and 96h of incubation. At 24h, 48h, 72h and 96h incubation period, the Morphological endpoint of zebrafish embryos was observed following the parameters explained by (Nagel, 2002), Lethal (coagulation, tail not detached, and no heart-beat) and Teratogenic effects (malformation of head, heart and tail, deformity of yolk, and growth retardation) were examined and images were taken using stereomicroscope. The Toxicity of the drugs is visualized by Percentage hatchability, Mortality and Heartbeat rate. The heartbeat rate was calculated by counting the heartbeat for 1 minute.

The percentage of the hatchability is measured by,

$$\% \text{ Hatchability} = \frac{\text{Number of Hatched embryos}}{\text{Total number of embryos}} \times 100$$

The percentage of mortality is measured by,

$$\% \text{ Mortality} = \frac{\text{Number of Dead embryos}}{\text{Total number of embryos}} \times 100$$

#### **4.3.3 Measurement of ROS generation in zebrafish**

The generation of intracellular ROS levels in Zebrafish larvae was detected using an oxidation-sensitive fluorescent probe DCFH-DA (2', 7'-dichlorodihydrofluorescein diacetate). The embryos were treated with the concentrations axitinib (10µg/mL), monacolin-x (10µg/mL) as individual and axitinib+monacolin-x (10µg/mL+10µg/mL) combination. ROS production in zebrafish larvae was assessed at 96 hrs and at the end of the exposure the larvae was stained with DCFH-DA in concentration of (20µg/mL) and incubated at room temperature in dark for 1h. After incubation, the embryos were rinsed with fresh E3 medium. The fluorescence intensity of zebrafish larvae was captured using a fluorescent stereomicroscope. Whole-body fluorescence was quantified using Image J software (USA) (D'Iglio et al., 2023).

#### **4.4 STATISTICAL ANALYSIS**

All results were presented as mean± standard error (SEM) and mean± standard deviation (SD) from triplicate experiments performed in a parallel manner. All statistical calculations were performed using the computer program GraphPad Prism-8.2 (GraphPad Software, CA, USA). All comparisons were made relative to untreated controls, axitinib and monacolin-x individual treatment. A statistically significant difference was considered at  $P \leq 0.05$ . The tests that were performed are in detailed below (**Tab 4.3**).

**TABLE 4.3 statistical analyses for experiments**

| <b>S.NO</b> | <b>EXPERIMENT</b>                 | <b>STATISTICAL TEST</b>                          |
|-------------|-----------------------------------|--------------------------------------------------|
| 1           | MTT Assay                         | Two way ANOVA (Tukey's multiple comparison test) |
| 2           | DCFH staining                     | One way ANOVA (Tukey's multiple comparison test) |
| 3           | PI staining                       | One way ANOVA (Tukey's multiple comparison test) |
| 4           | AO/EB Staining                    | Chi-square Test                                  |
| 5           | DPPH Assay                        | Two way ANOVA (Tukey's multiple comparison test) |
| 6           | qRT-PCR                           | One way ANOVA (Tukey's multiple comparison test) |
| 7           | Embryo mortality and hatchability | One way ANOVA (Tukey's multiple comparison test) |
| 8           | Embryo heartbeat rate             | Unpaired T-Test                                  |

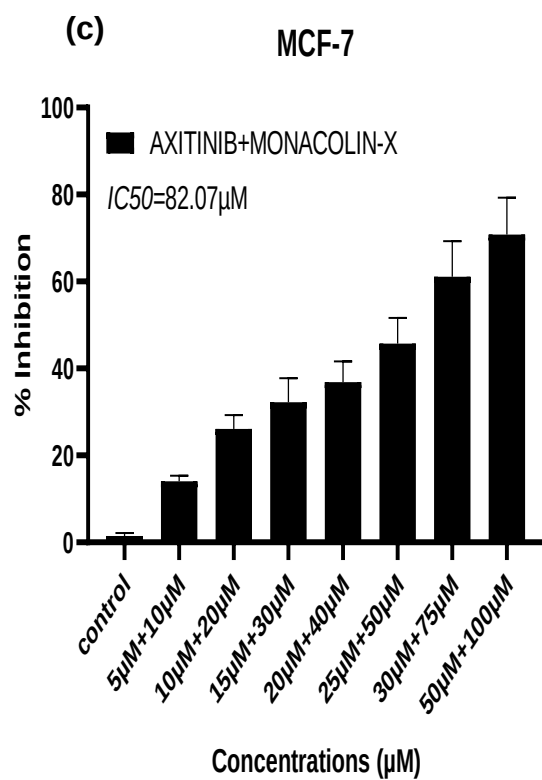
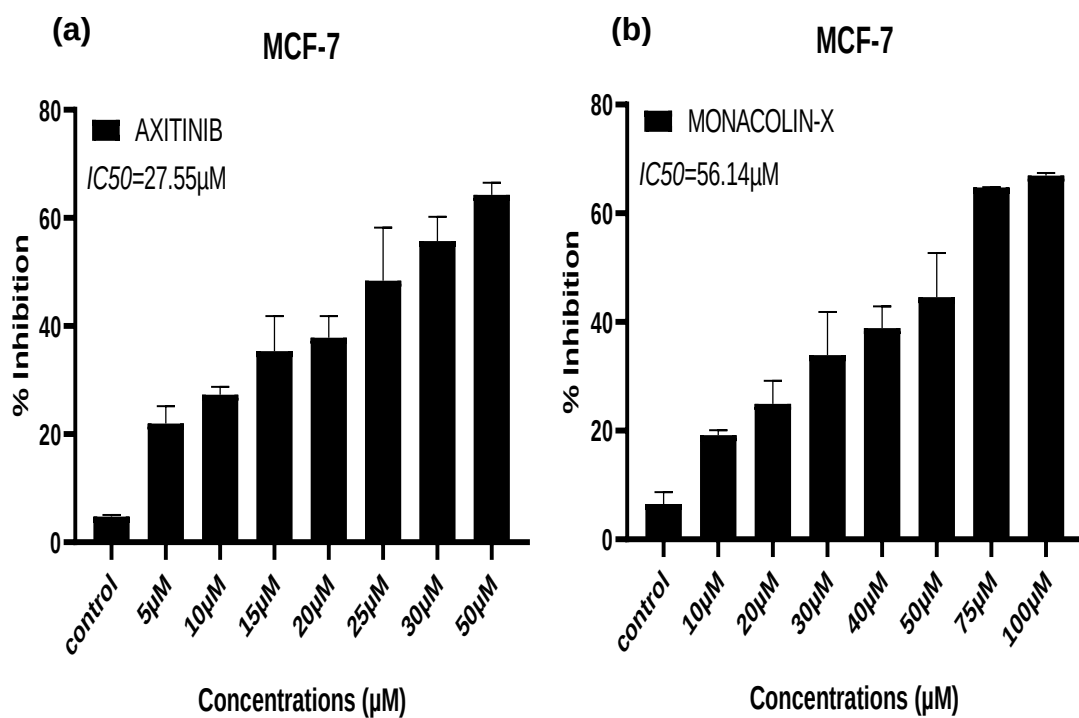
## CHAPTER 5

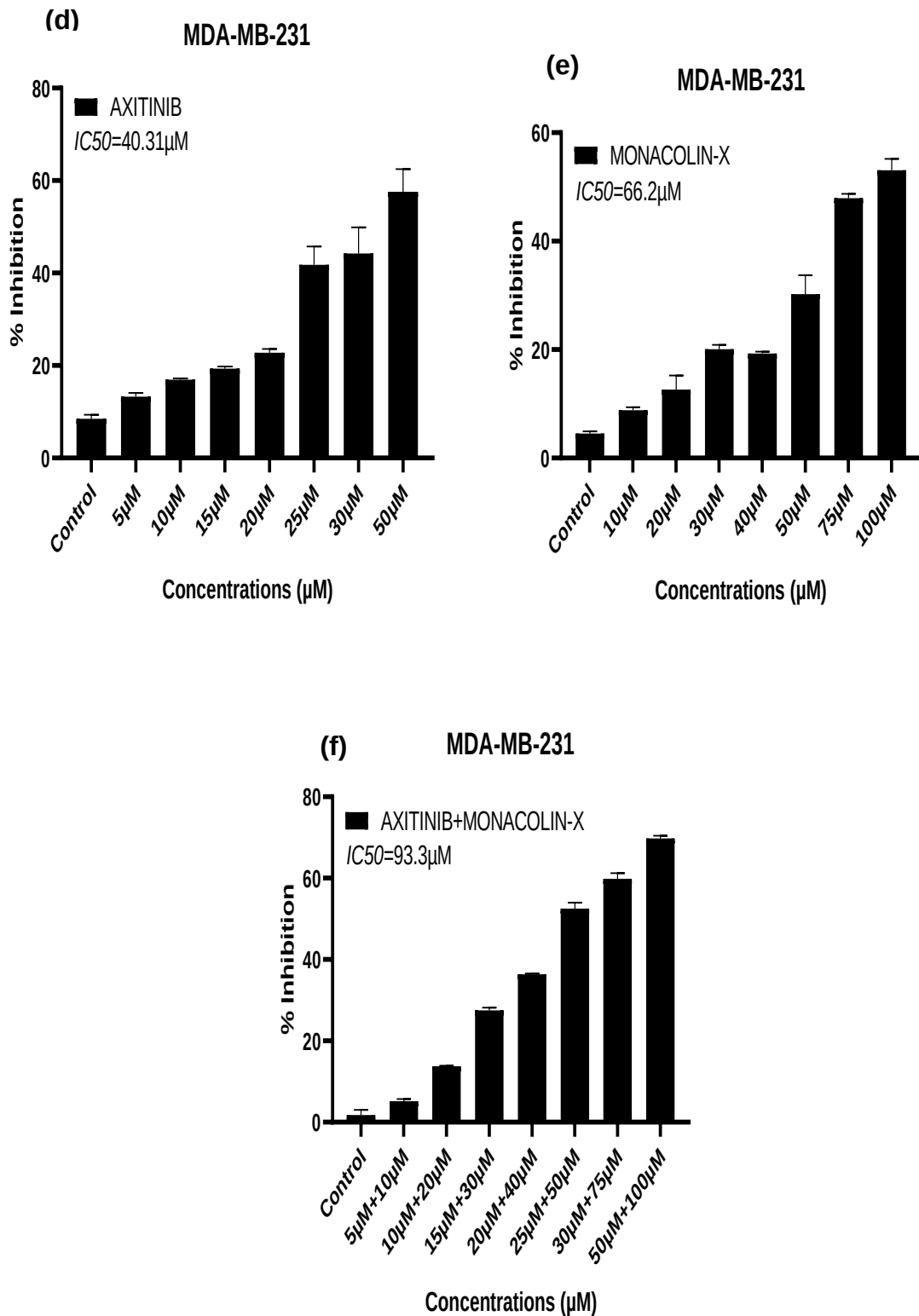
### RESULTS AND DISCUSSION

#### 5.1 IN VITRO STUDIES

##### ***5.1.1 Cytotoxicity effect of axitinib and monacolin-x combination treatment***

To examine the effect of axitinib and monacolin-x as combination treatment on the growth of BC cells MCF-7 and MDA-MB-231 was measured by MTT assay. MCF-7 and MDA-MB-231 cells were treated with different concentrations of axitinib alone (5 $\mu$ M, 10 $\mu$ M, 15 $\mu$ M, 20 $\mu$ M, 25 $\mu$ M, 30 $\mu$ M, 50 $\mu$ M), monacolin-x alone (10 $\mu$ M, 20 $\mu$ M, 30 $\mu$ M, 40 $\mu$ M, 50 $\mu$ M, 75 $\mu$ M, 100 $\mu$ M) and axitinib+monacolin-x (5 $\mu$ M+10 $\mu$ M, 10 $\mu$ M+20 $\mu$ M, 15 $\mu$ M+30 $\mu$ M, 20 $\mu$ M+40 $\mu$ M, 25 $\mu$ M+50 $\mu$ M, 30 $\mu$ M+75 $\mu$ M, 50 $\mu$ M+100 $\mu$ M) in combination for 24h. Axitinib alone, monacolin-x alone and axitinib+monacolin-x combination treatment inhibited cell viability in a dose-dependent manner on MCF-7 and MDA-MB-231 cells. Compared to axitinib and monacolin-x individual treatment combination of axitinib+monacolin-x exerted a stronger inhibition of cell growth in a dose-dependent manner on MDA-MB-231 and MCF-7 cells. The % of inhibition in axitinib+monacolin-x treatment is 14.07, 26.1, 32.2, 36.8, 45.7, 61.1, and 70.8 in 5+10, 10+20, 15+30, 20+40, 25+50, 30+75, and 50+100  $\mu$ M concentrations respectively and IC<sub>50</sub> was found to be 82.7 $\mu$ M on MCF-7 cells (**Fig 5.1c**) and the % of inhibition in MDA-MB-231 cells, axitinib+monacolin-x treatment is 5.1, 13.7, 27.4, 36.3, 52.4, 59.8, and 69.7 in 5+10, 10+20, 15+30, 20+40, 25+50, 30+75, and 50+100  $\mu$ M concentrations respectively and IC<sub>50</sub> was found to be 93.3 $\mu$ M (**Fig 5.1f**). Thus the axitinib+monacolin-x combination treatment showed significant enhancement in killing breast cancer cells compared to axitinib and monacolin-x individual treatment.





**Figure 5.1 Percentage inhibition of axitinib, monacolin-x individual treatment and axitinib+monacolin-x combination treatment on MCF-7 and MDA-MB-231 cells. Axitinib+Monacolin-x combination shows significant cell inhibition compared**

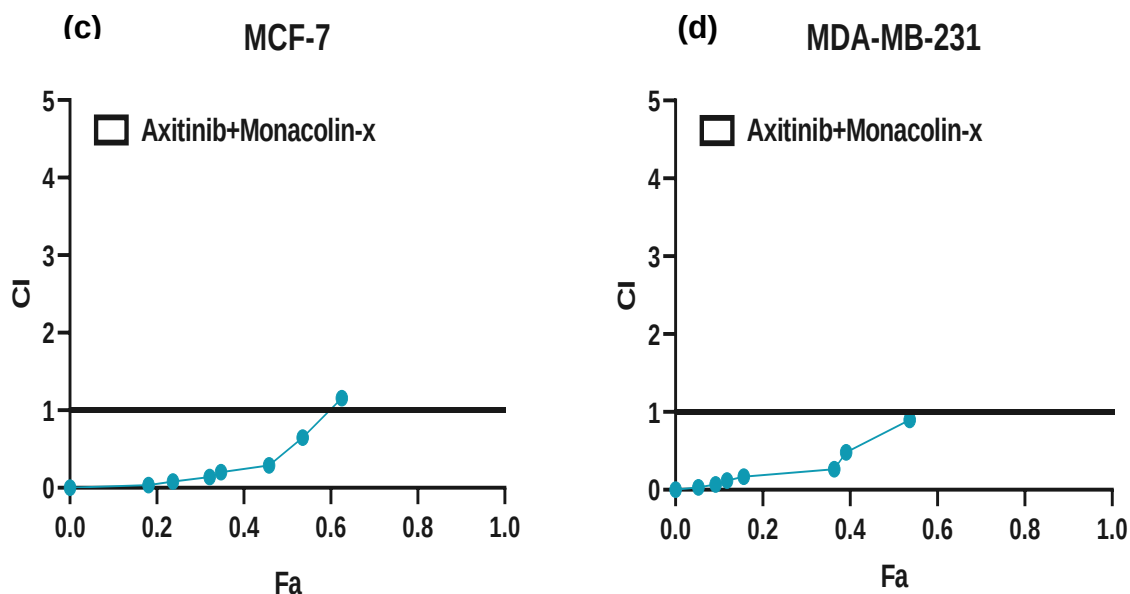
to axitinib and monacolin-x individual treatment in MDA-MB-231 cells whereas in MCF-7 cells, axitinib+monacolin-x combination treatment shows no significant difference in cell inhibition when compared to axitinib and monacolin-x individual treatment but there is 4% increase in cell inhibition. The values were expressed in mean $\pm$ SD (n=3); statistical analysis was performed using Two way ANOVA followed by Tukey's multiple comparison test, p value>0.05 vs. axitinib and monacolin-x in MCF-7 cells then p value<0.05 vs. axitinib and monacolin-x in MDA-MB-231 cells.

#### 5.1.1.1 Combination effect of Axitinib and Monacolin-x

The combination effect of axitinib and monacolin-x on MCF-7 and MDA-MB-231 cells were evaluated by calculating combination index (CI). In MCF-7 cells, synergistic effect (CI<1) of axitinib and monacolin-x was observed when Fa value was <0.5 and Fa value >0.5 showed additive effect (CI=1) (**Tab 5.1a**). Axitinib and Monacolin-x showed synergistic effect (CI<1) in MDA-MB-231 cells when Fa value was <0.5 (**Tab 5.1b**).

**TABLE 5.1 Fa and CI data of axitinib and monacolin-x for MCF-7 and MDA-MB-231 cells.**

| (a) MCF-7              |                        | (b) MDA-MB-231         |                        |
|------------------------|------------------------|------------------------|------------------------|
| Fraction affected (Fa) | Combination Index (CI) | Fraction affected (Fa) | Combination Index (CI) |
| 0                      | 0                      | 0                      | 0                      |
| 0.180792               | 0.036678               | 0.05245                | 0.032708               |
| 0.236733               | 0.081814               | 0.092219               | 0.070024               |
| 0.321124               | 0.139094               | 0.118156               | 0.119563               |
| 0.34753                | 0.202532               | 0.156196               | 0.168131               |
| 0.458262               | 0.289243               | 0.363689               | 0.263606               |
| 0.535136               | 0.646019               | 0.390778               | 0.480455               |
| 0.625                  | 1.15442                | 0.536599               | 0.897939               |



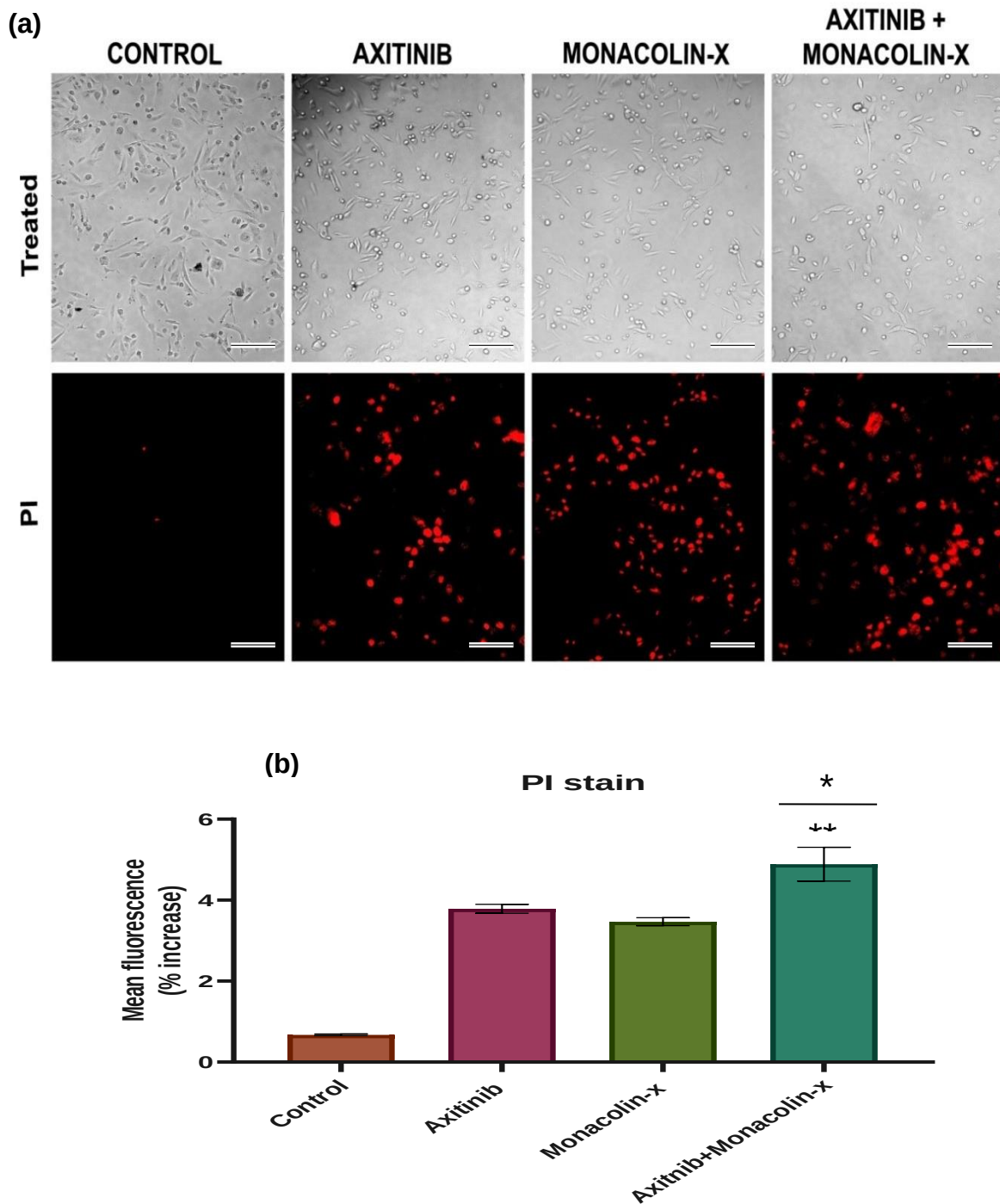
**Figure 5.2 Fa vs CI plot of axitinib+monacolin-x in MCF-7 and MDA-MB-231 cells. (c)** synergistic effect ( $CI < 1$ ) when  $Fa < 0.5$  and additive effect ( $CI = 1$ ) when  $Fa > 0.5$  between axitinib and monacolin-x in MCF-7 cells. **(d)** Synergistic effect ( $CI < 1$ ) when  $Fa < 0.5$  between axitinib and monacolin-x in MDA-MB-231 cells.

### 5.1.2 Evaluation of apoptosis induced by axitinib and monacolin-x combination treatment

#### 5.1.2.1 Propidium Iodide (PI) Staining

Propidium iodide (PI) staining is a valuable method used to show the morphological changes under a fluorescence microscope and to distinguishing between cell death mediated by apoptosis or necrosis. Morphological observation of apoptotic cells are distinguished by cell shrinkage, chromatin condensation, membrane blebbing, DNA fragmentation and formation of small apoptotic bodies. PI was used to study the apoptotic effect of axitinib and monacolin-x as individual and as a combination treatment on MDA-MB-231 cells. The cells were treated at IC50 concentration of axitinib, monacolin-x as individual and axitinib+monacolin-x combination for 24h. The percentage of apoptotic nuclei after treatment with IC50 concentration of axitinib+monacolin-x considerably increased when compared to axitinib, monacolin-x individual treatment and control (untreated cells) in MDA-MB-231 cells (**Fig 5.3a**). The control cells were intact and spindle shaped. Whereas; axitinib, monacolin-x and axitinib+monacolin-x treated cells show apoptosis

features including shrinkage of the cells, nuclear condensation, fragmented nuclei formation and increased amount of apoptotic body formation.

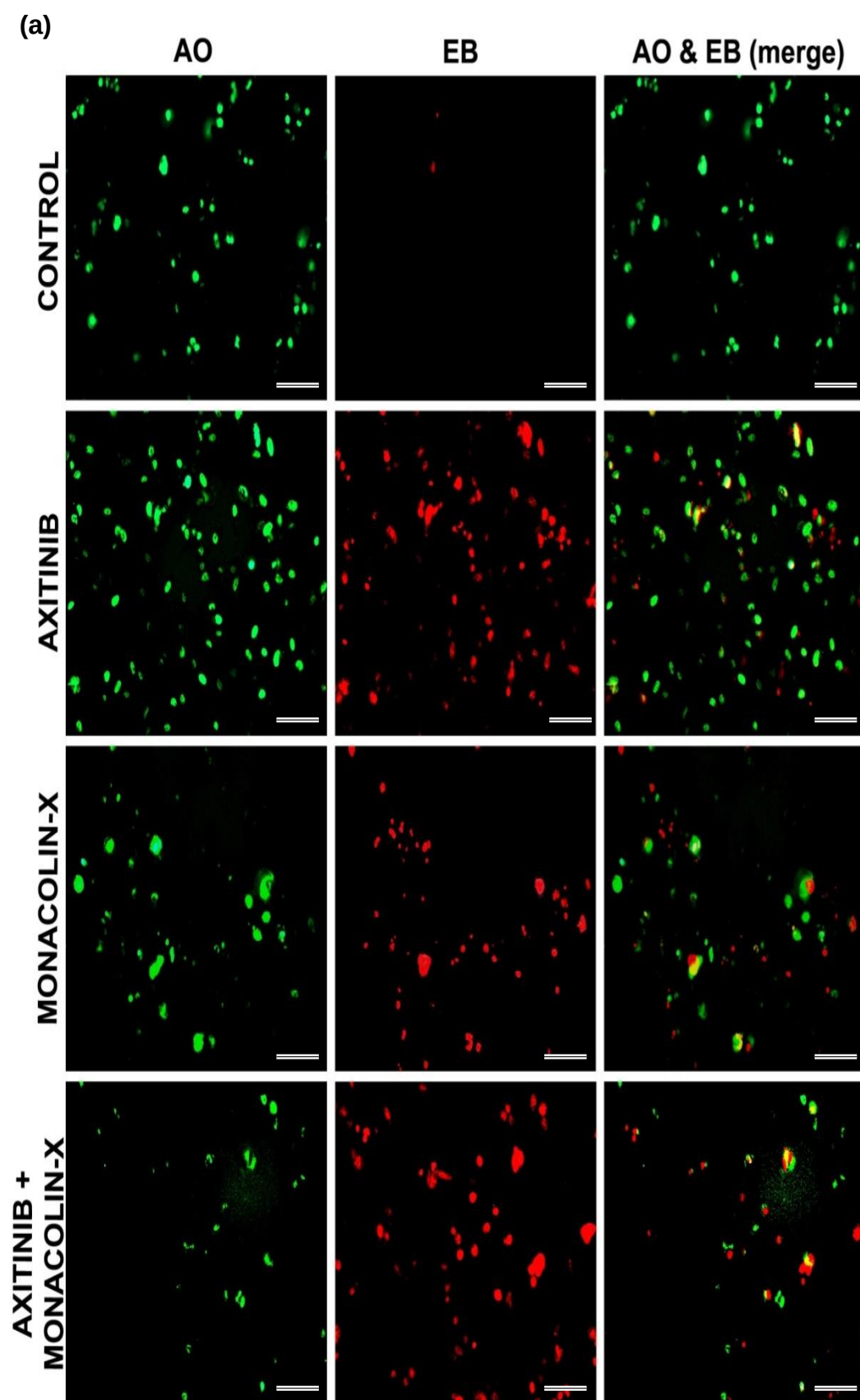


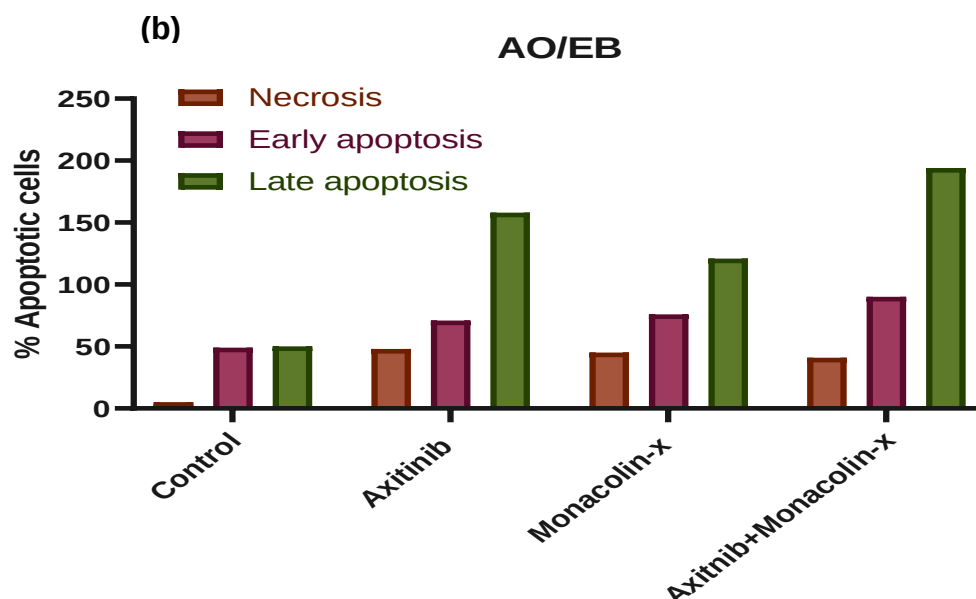
**Figure 5.3 Propidium Iodide staining to assess cellular morphology.** (a) PI staining on MDA-MB-231 cells after 24 hr treatment with IC50 concentration of axitinib and monacolin-x as individual and as combination reveals nuclear morphology. Control MDA-MB-231 cells depict normal nuclei with less uniform red fluorescence and with minimal debris. Axitinib alone and monacolin-x alone

treated cells showed shrinkage of the nucleus with deformed morphology, chromatin condensation. Axitinib+Monacolin-x combination treated cells showed a discernible change in the nuclear morphology with increased deformed nuclei. **(b)** Axitinib+Monacolin-x combination showed significant increase in number of apoptotic cells (380) measured by Image J software with mean fluorescence intensity (4.8) compared to axitinib, monacolin-x individual treatment and control. The values were expressed in mean $\pm$ SD (n=2); statistical analysis was performed using one way ANOVA followed by Tukey's multiple comparison test, \*indicates p value<0.05 vs. axitinib and monacolin-x and \*\*indicates p value<0.01 vs. control.

#### 5.1.2.2 Acridine Orange and Ethidium Bromide Dual Staining (AO/EB)

Acridine Orange/Ethidium Bromide dual staining was performed to assess the cellular morphological alterations and apoptosis inducing activity of axitinib and monacolin-x as combination and individual treatment on MDA-MB-231 cells. Dual AO/EB staining enables to distinguish between necrotic cells, early apoptosis cells and late apoptosis cells. The AO/EB stained cells were characterized to be viable (light green), early apoptotic (yellow fluorescence and condensed chromatin), late apoptotic (orange fluorescence) and nonviable cells (red colored fluorescence). MDA-MB-231 cells were treated at IC<sub>50</sub> concentration of axitinib, monacolin-x as individual and axitinib+monacolin-x combination for 24h. axitinib+monacolin-x treatment cells showed significantly increased level of apoptotic cells when compared to axitinib, monacolin-x individual treatment and control (untreated cells) **(Fig 5.4a)**. In control MDA-MB-231 cells, no significant apoptosis was noticed, where the live cells produced green fluorescence with normal cytoplasm and nuclei morphology. Whereas; axitinib, monacolin-x and axitinib+monacolin-x treated cells have shown membrane blebbing, condensed nuclei, and apoptotic bodies with appearance of yellowish-green cells indicating early apoptotic cells with nuclear margination and chromatin condensation and late apoptotic cells with bright orange color having condensed chromatin in the nucleus and then necrotic cells with red color deformed nuclei.





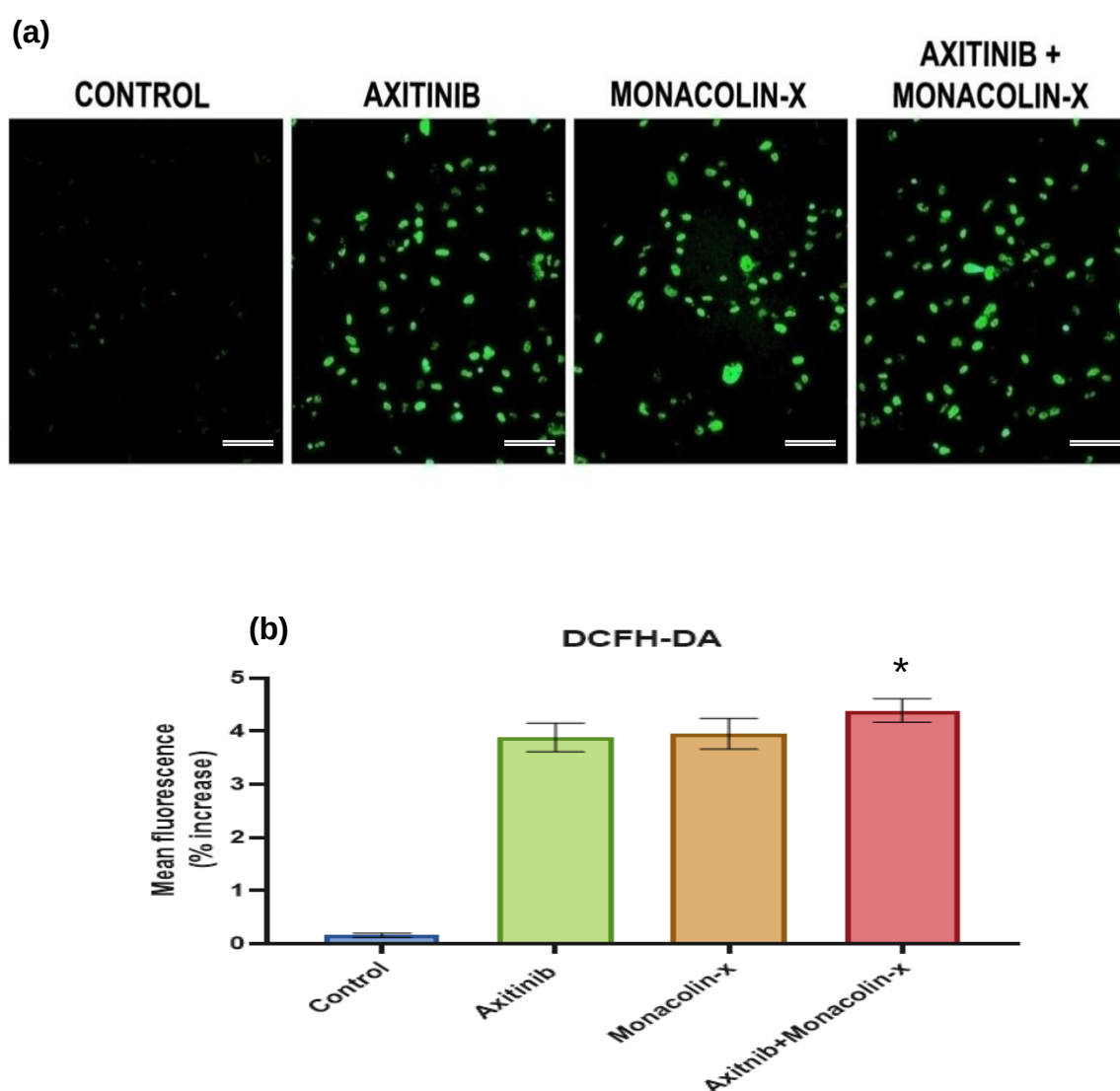
**Figure 5.4 Acridine Orange and Ethidium Bromide dual staining on MDA-MB-231 cells to differentiate early apoptotic, late apoptotic and necrotic cells. (a)**

Apoptosis is evidenced by AO/EB staining of MDA-MB-231 cells under indicated treatment condition *in vitro*. The last row represents the merged images of AO and EB. Control untreated MDA-MB-231 cells excluded ethidium bromide and their nuclei appeared bright green with intact structure. Axitinib, monacolin-x individual treatment and axitinib+monacolin-x combination treatment MDA-MB-231 cells showed early apoptotic cells (light yellow color nuclei), late apoptotic cells (bright orange nuclei) and necrotic cells (red-colored apoptotic bodies) at 20X magnification. **(b)** Axitinib+Monacolin-x combination showed a significant increase in number of apoptotic cells measured by Image J software with around 80% of the cells in early apoptosis and 194% in the late apoptosis stage compared to axitinib, monacolin-x individual treatment and control. The values were expressed in mean $\pm$ SEM; statistical analysis was performed using Chi square test,  $p$  value $<0.05$  vs. axitinib, monacolin-x and control.

### **5.1.3 Axitinib and Monacolin-x combination treatment modulates intracellular ROS production in MDA-MB-231 cells**

Increase in ROS with antitumor drugs treatment was exposed to induce DNA damage and apoptosis by reducing the mitochondrial membrane potential with subsequent release of cytochrome C, activation of caspase3, increased Bax expression and decreased Bcl2 expression. MDA-MB-231 cells were treated at

IC50 concentration of axitinib, monacolin-x as individual and axitinib+monacolin-x combination for 24h. To investigate whether the axitinib and monacolin-x combination treatment induces oxidative stress, DCFH-DA fluorogenic dye was used, which emits green fluorescence after oxidized to DCF. The axitinib+monacolin-x combination treatment shows significantly increased levels of ROS generation compared to axitinib, monacolin-x individual treatment and control (untreated cells) in MDA-MB-231 cells (**Fig 5.5a**). The control MDA-MB-231 cells had slight basal intracellular ROS whereas, the fluorescence amount of DCF in axitinib, monacolin-x and axitinib+monacolin-x treatment cells amplified significantly.

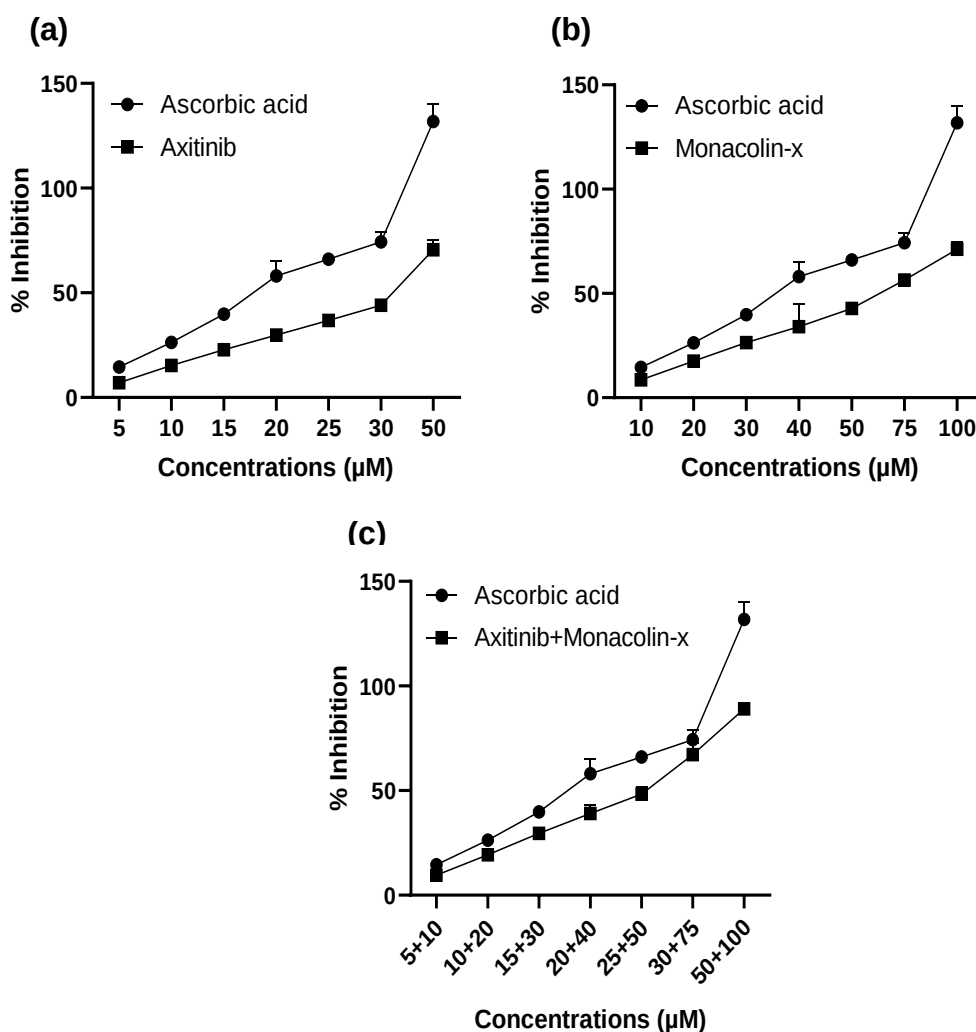


**Figure 5.5 Evaluation of intracellular ROS by DCFH-DA assay. (a)** DCFH-DA staining on MDA-MB-231 cells after 24 hr treatment with IC50 concentration of axitinib and monacolin-x as individual and as combination reveals intracellular

ROS generation. Control MDA-MB-231 cells have depicted less ROS generation. Axitinib, monacolin-x alone and axitinib+monacolin-x combination showed significant ROS generation upon treatment. **(b)** Axitinib+Monacolin-x combination showed significant increase in intracellular levels of ROS generation measured by Image J software with mean fluorescence (4.38) compared to control and shows no significant difference in ROS generation compared to axitinib and monacolin-x individual treatment but there is 0.4% increase in levels of ROS generation. The values were expressed in mean $\pm$ SD (n=2); statistical analysis was performed using one way ANOVA followed by Tukey's multiple comparison test, \*indicates p value<0.05 vs. control and p value>0.05 vs. axitinib and monacolin-x.

#### **5.1.4 DPPH free radical scavenging assay**

Free radical scavenging activity of axitinib and monacolin-x as combination was estimated using DPPH scavenging assay. DPPH radical is scavenged by antioxidants through the donation of protons resulting in the reduced DPPH. axitinib alone (5 $\mu$ M, 10 $\mu$ M, 15 $\mu$ M, 20 $\mu$ M, 25 $\mu$ M, 30 $\mu$ M, 50 $\mu$ M), monacolin-x alone (10 $\mu$ M, 20 $\mu$ M, 30 $\mu$ M, 40 $\mu$ M, 50 $\mu$ M, 75 $\mu$ M, 100 $\mu$ M) and axitinib+monacolin-x (5 $\mu$ M+10 $\mu$ M, 10 $\mu$ M+20 $\mu$ M, 15 $\mu$ M+30 $\mu$ M, 20 $\mu$ M+40 $\mu$ M, 25 $\mu$ M+50 $\mu$ M, 30 $\mu$ M+75 $\mu$ M, 50 $\mu$ M+100 $\mu$ M) in combination was added at different concentrations with standard control ascorbic acid (5  $\mu$ l, 10  $\mu$ l, 15  $\mu$ l, 20  $\mu$ l, 25  $\mu$ l, 30  $\mu$ l, 50  $\mu$ l). The radical scavenging activity of DPPH by axitinib+monacolin-x combination was increased when compared to axitinib, monacolin-x individual treatment and decreased when compared to ascorbic acid. Axitinib and monacolin-x alone showed 50% DPPH free radicals scavenging activity approximately at the concentrations 30 $\mu$ M and 50 $\mu$ M respectively (**Fig 5.6a,b**). Axitinib+monacolin-x combination scavenged 50% DPPH free radicals approximately at the concentration (25 $\mu$ M+50 $\mu$ M) which is equal to the IC<sub>50</sub> concentration (**Fig 5.6c**). Overall, DPPH radical scavenging activity was found higher in axitinib+monacolin-x combination confirming their antioxidant activity and it was found almost similar to standard ascorbic acid.



**Figure 5.6** DPPH free radical scavenging activity of axitinib and monacolin-x as individual and combination treatment with standard control ascorbic acid. The values were expressed in mean $\pm$ SEM; statistical analysis was performed using two way ANOVA followed by Tukey's multiple comparison test,  $p$  value $<0.05$  vs. axitinib, monacolin-x and standard control ascorbic acid.

### 5.1.5 Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)

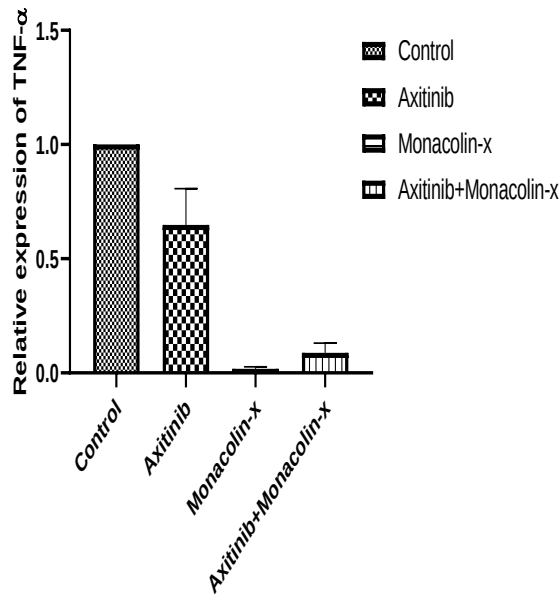
The efficiency of axitinib and monacolin-x combination was evaluated by assessing the mRNA expression of Pro-angiogenic and pro- inflammation factors tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-6 (IL-6), interleukin 1 beta (IL-1 $\beta$ ) and nuclear factor-kappa B (NF- $\kappa$ B). Dysregulation of these factors contributes to disease progression, angiogenesis, and resistance to treatment in TNBC. GAPDH, a housekeeping gene, was used as an endogenous control for the qRT-PCR analysis. The mRNA expression of all the genes TNF- $\alpha$ , IL-6, IL-1 $\beta$  and NF- $\kappa$ B

was significantly downregulated after treatment with IC50 concentration of axitinib and monacolin-x as individual and combination when compared to control **(Fig 5.7a-d)**. Relative mRNA expression of the gene TNF- $\alpha$  after axitinib+monacolin-x treatment was significantly downregulated with fold change of 0.08% when compared to axitinib individual treatment fold change 0.64% and upregulated when compared to monacolin-x individual treatment fold change 0.01% **(Tab 5.2) (Fig 5.7a)**. Relative mRNA expression of the gene IL-6 after axitinib+monacolin-x treatment was significantly downregulated with fold change of 0.3% when compared to axitinib and monacolin-x individual treatment with fold changes of 0.61% and 0.73% respectively **(Tab 5.2) (Fig 5.7b)**. Relative mRNA expression of the gene IL-1 $\beta$  after axitinib+monacolin-x treatment was downregulated with fold change of 0.05% when compared to axitinib and monacolin-x individual treatment with fold changes of 0.09% and 0.08% respectively **(Tab 5.2) (Fig 5.7c)**. Relative mRNA expression of the gene NF- $\kappa$ B after axitinib+monacolin-x treatment was significantly downregulated with fold change of 0.1% when compared to monacolin-x with fold changes of 0.36% and downregulated when compared to axitinib individual treatment with fold change 0.18% **(Tab 5.2) (Fig 5.7d)**.

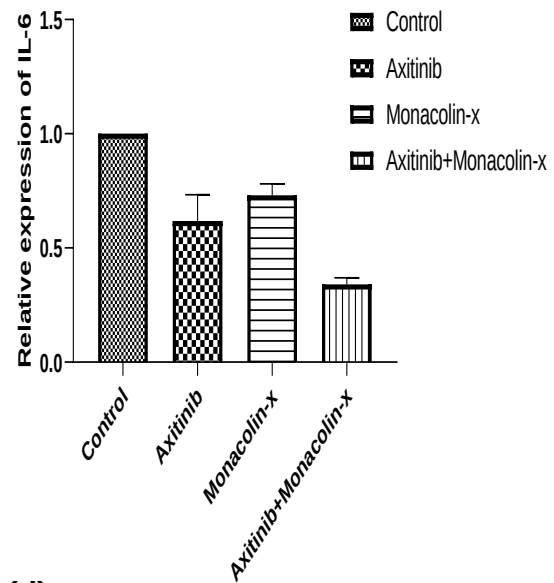
**TABLE 5.2 Fold changes in target genes**

| S.NO | TARGET GENE     | FOLD CHANGE |             |                      |
|------|-----------------|-------------|-------------|----------------------|
|      |                 | AXITINIB    | MONACOLIN-X | AXITINIB+MONACOLIN-X |
| 1    | Control (GAPDH) | 1.000000    | 1.000000    | 1.000000             |
| 2    | TNF- $\alpha$   | 0.648131    | 0.0185845   | 0.0886123            |
| 3    | IL-6            | 0.618316    | 0.731086    | 0.340744             |
| 4    | IL-1 $\beta$    | 0.0944892   | 0.0821828   | 0.0522237            |
| 5    | NF- $\kappa$ B  | 0.180044    | 0.368629    | 0.109594             |

**(a)** Expression of TNF- $\alpha$  on MDA-MB-231

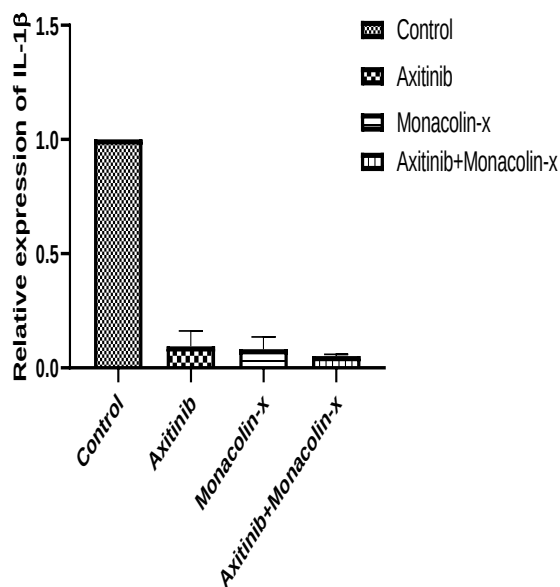


**(b)** Expression of IL-6 on MDA-MB-231



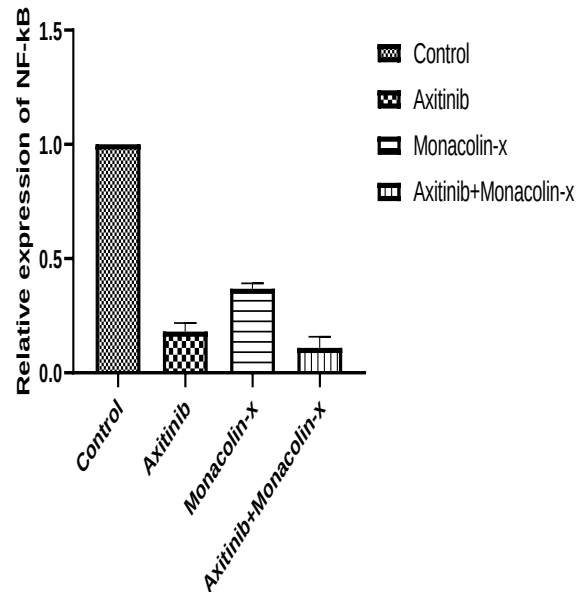
**(c)**

Expression of IL-1 $\beta$  on MDA-MB-231



**(d)**

Expression of NF- $\kappa$ B on MDA-MB-231



**Figure 5.7 Relative mRNA expression of genes TNF- $\alpha$ , IL-6, IL-1 $\beta$  and NF- $\kappa$ B by qRT-PCR. (a)** Relative mRNA expression of gene TNF- $\alpha$  after axitinib+monacolin-x treatment showed significant difference when compared to control, axitinib individual treatment and no significant difference when compared to monacolin-x individual treatment. **(b)** Relative mRNA expression of gene IL-6 after axitinib+monacolin-x treatment showed significant difference when compared to control, axitinib and monacolin-x individual treatment. **(c)** Relative mRNA

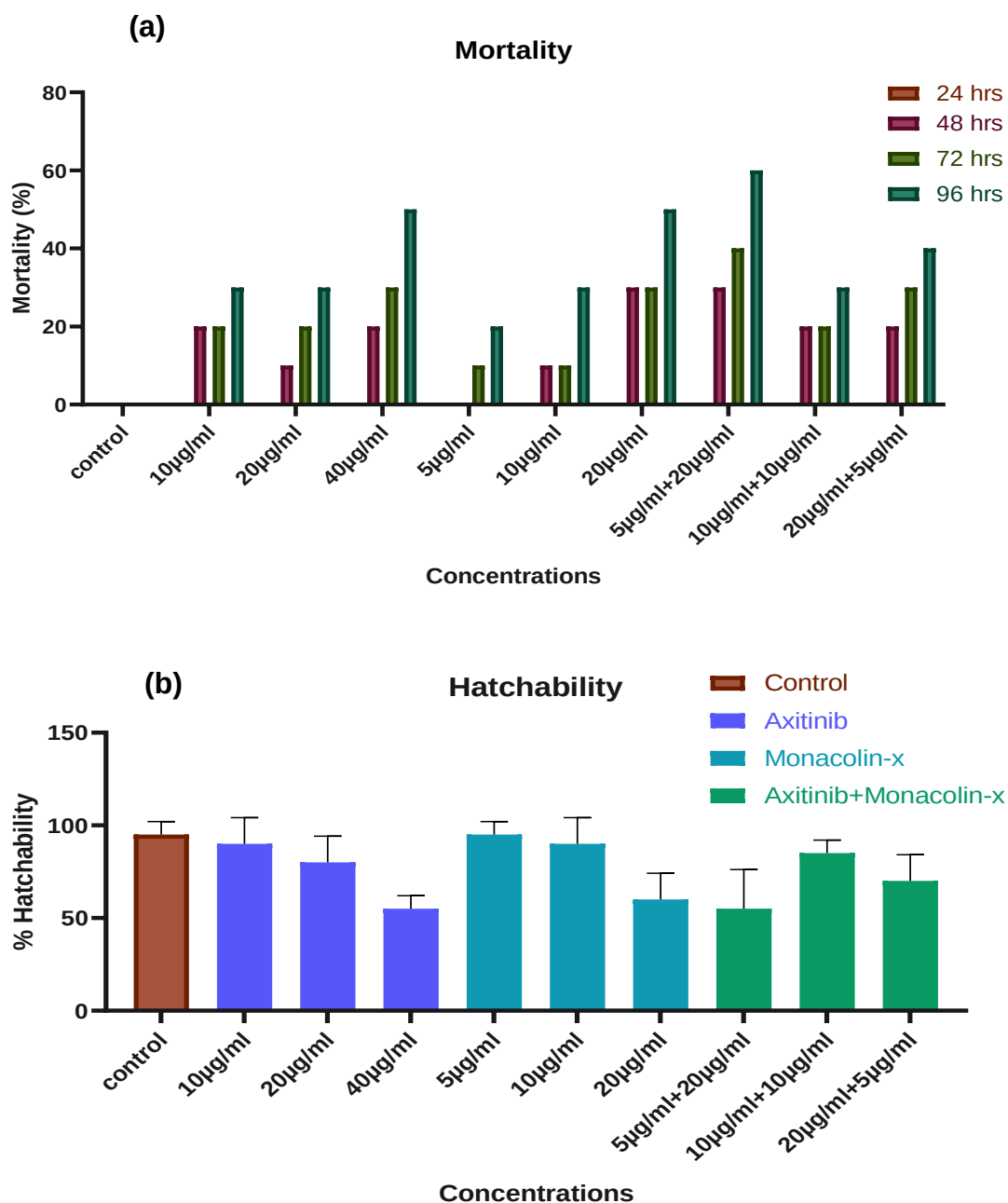
expression of gene *IL-1 $\beta$*  after axitinib+monacolin-x treatment showed significant difference when compared to control and no significant difference when compared to axitinib and monacolin-x individual treatment but there is a trend in decrease of fold change about 0.05%. **(d)** Relative mRNA expression of gene *NF- $\kappa$ B* after axitinib+monacolin-x treatment showed significant difference when compared to control, monacolin-x individual treatment and no significant difference when compared to axitinib individual treatment but there is a trend in decrease of fold change about 0.1%. The values were expressed in mean $\pm$ SD (n=2); statistical analysis was performed using one way ANOVA followed by Tukey's multiple comparison test, *p* value<0.05 and *p* value<0.01 vs. axitinib, monacolin-x and control.

## 5.2 IN VIVO STUDIES

### 5.2.1 Embryo Toxic Effect of Axitinib and Monacolin-x on Zebrafish Embryos

Embryo toxicity of zebrafish embryos exposed to various concentrations of axitinib and monacolin-x as individual and combination treatment was assessed by percentage of mortality and hatchability of embryos at different time intervals (24-96 hrs). The embryos were treated at axitinib (10 $\mu$ g/mL, 20 $\mu$ g/mL and 40 $\mu$ g/mL), monacolin-x (5 $\mu$ g/mL, 10 $\mu$ g/mL and 20 $\mu$ g/mL) and axitinib+monacolin-x (5 $\mu$ g/mL+20 $\mu$ g/mL, 10 $\mu$ g/mL+10 $\mu$ g/mL and 20 $\mu$ g/mL+5 $\mu$ g/mL) concentrations. Mortality was determined as coagulation and no visual heartbeat of embryos. After 24 hrs of incubation no mortality was observed in embryos exposed to axitinib and monacolin-x as individual and combination. After 48, 72 and 96 hours of incubation, axitinib and monacolin-x as combination showed increased mortality about 60% at (5 $\mu$ g/mL + 20 $\mu$ g/mL) and decreased mortality about 30% and 40% at (10 $\mu$ g/mL + 10 $\mu$ g/mL and 20 $\mu$ g/mL + 5 $\mu$ g/mL) concentration. Axitinib alone showed increased mortality about 50% at (40 $\mu$ g/mL) and decreased mortality about 30% at (10 $\mu$ g/mL, 20 $\mu$ g/mL) concentrations. Monacolin-x alone showed increased mortality about 50% at (20 $\mu$ g/mL) and decreased mortality about 20% and 30% at (5 $\mu$ g/mL and 10 $\mu$ g/mL) concentrations and in control no mortality was observed from 24-96 hours (**Fig 5.8a**). The hatchability of embryos is observed from 48 hrs and control shows 100% hatchability. The hatchability of embryos exposed to axitinib and monacolin-x combination showed significant increased

hatching rate about 85% at (10µg/mL + 10µg/mL) and decreased hatching rate about 55% and 70% at (5µg/mL + 20µg/mL and 20µg/mL + 5µg/mL) concentrations respectively. Axitinib alone showed increased hatching rate about 90% and 80% at (10µg/mL + 20µg/mL) and decreased hatching rate about 55% at (40µg/mL) concentration. Monacolin-x as individual showed increased hatching rate about 95% and 90% at (5µg/mL and 10µg/mL) concentrations respectively (Fig 5.8b).

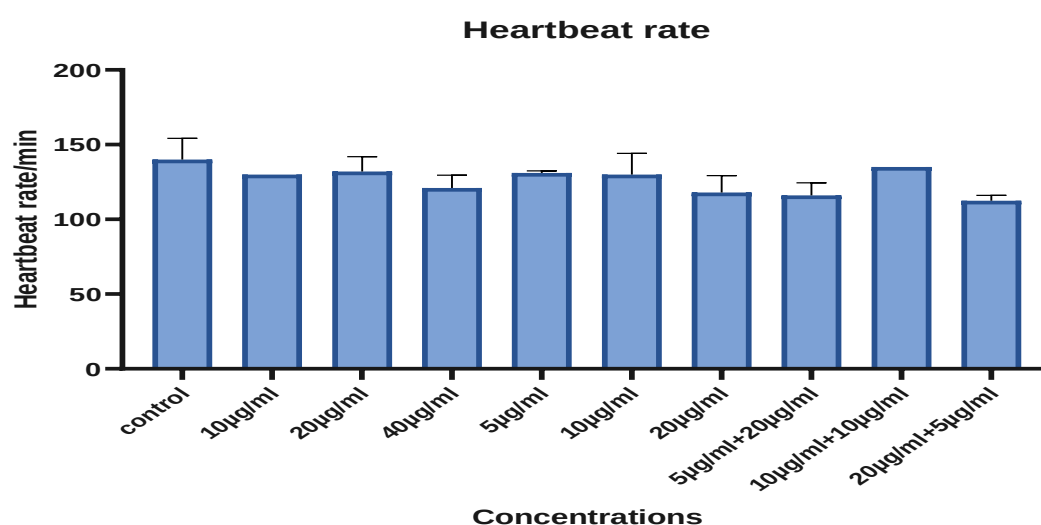


**Figure 5.8 (a)** Percentage mortality of embryos exposed to various concentrations of axitinib (10µg/mL, 20µg/mL and 40µg/mL), monacolin-x (5µg/mL, 10µg/mL and

20µg/mL) and axitinib+monacolin-x (5µg/mL+20µg/mL, 10µg/mL+10µg/mL and 20µg/mL+5µg/mL) after different time periods 24, 48, 72 and 96hrs. **(b)** Percentage Hatchability of the embryos with different concentrations of axitinib (10µg/mL, 20µg/mL and 40µg/mL), monacolin-x (5µg/mL, 10µg/mL and 20µg/mL) and axitinib+monacolin-x (5µg/mL+20µg/mL, 10µg/mL+10µg/mL and 20µg/mL+5µg/mL) after 48 hrs. The values were expressed in mean±SEM; statistical analysis was performed using one way ANOVA followed by Sidak's multiple comparisons test, *p* value<0.05 vs. axitinib, monacolin-x and control for mortality and hatchability.

#### 5.2.1.1 Effect of Axitinib and Monacolin-x on Zebrafish Embryos Heartbeat Rate

Heartbeat rate of embryos were observed for 1 min after 48 hrs. Heartbeat rate of embryos at control was found to be 140/min. Among the treatment groups, axitinib and monacolin-x combination showed 135beats/min at (10µg/mL+10µg/mL), axitinib alone showed 132beats/min at (10µg/mL), and monacolin-x alone showed 131beats/min at (10µg/mL) was found statistically comparable with control embryos (**Fig 5.9**). However, embryos treated at axitinib (20µg/mL and 40µg/mL), monacolin-x (5µg/mL and 10µg/mL) and axitinib+monacolin-x combination (5µg/mL+20µg/mL and 20µg/mL+5µg/mL) concentrations significantly recorded lower heartbeat rate, which strongly denotes cardiotoxicity.

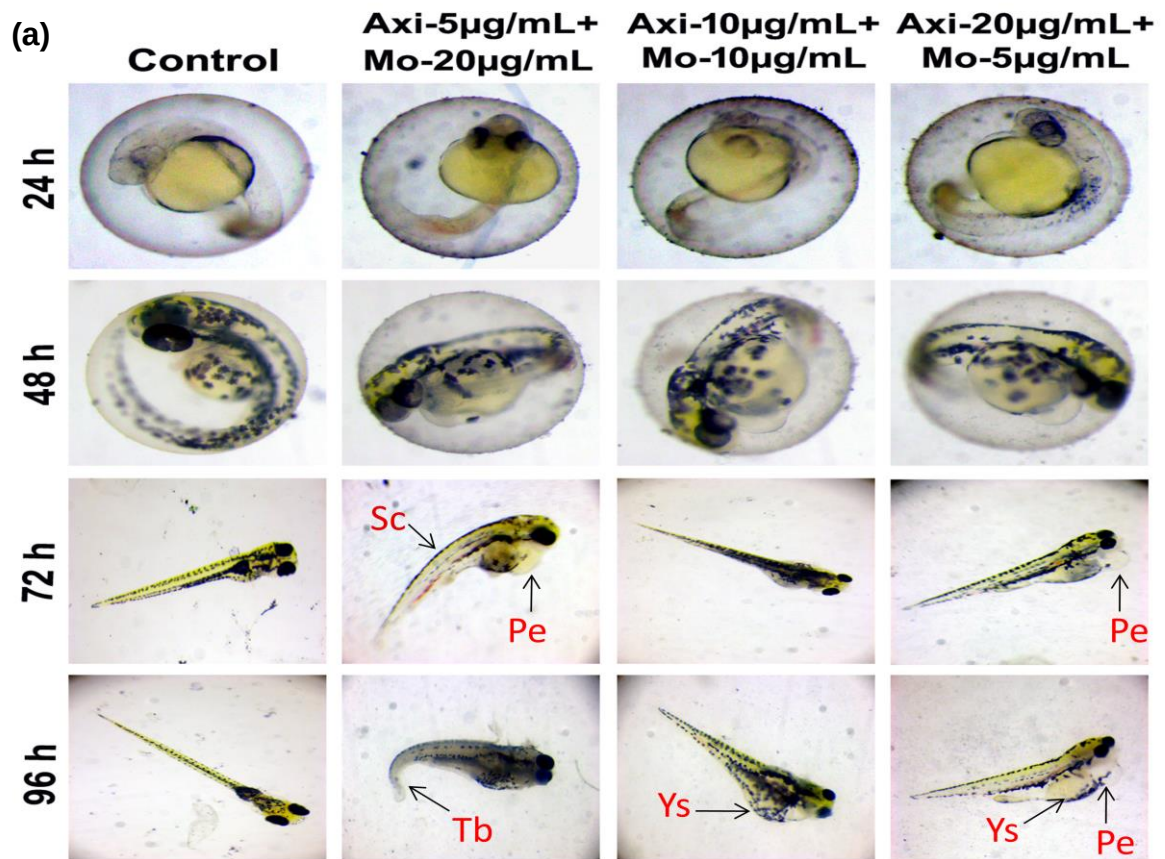


**Figure 5.9** Heartbeat rate of the zebrafish embryos after 48hrs in different concentrations of axitinib (10µg/mL, 20µg/mL and 40µg/mL), monacolin-x

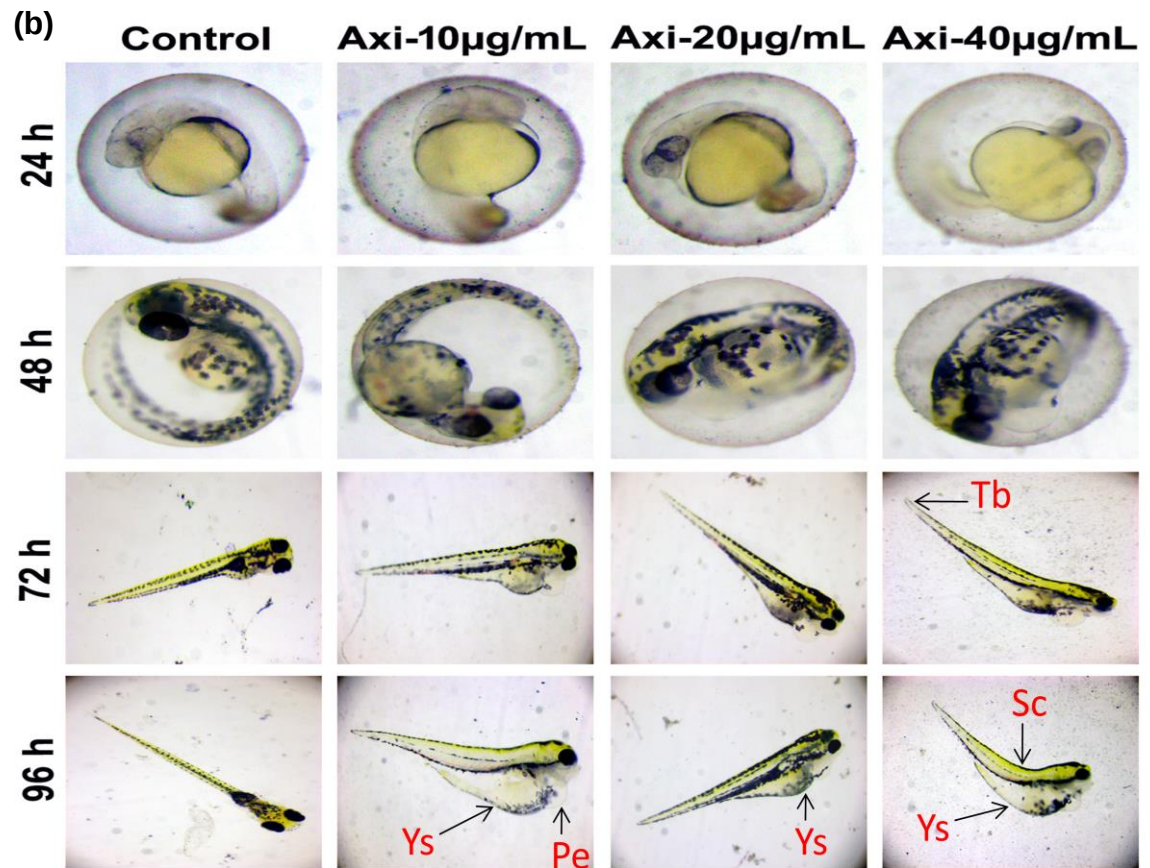
(5µg/mL, 10µg/mL and 20µg/mL) and axitinib+monacolin-x (5µg/mL+20µg/mL, 10µg/mL+10µg/mL and 20µg/mL+5µg/mL). The values were expressed in mean±SD (n=10) ; statistical analysis was performed using one sample t test, p value<0.01 vs. axitinib, monacolin-x and control.

### **5.2.2 Teratogenic activity of Axitinib and Monacolin-x on zebrafish embryos**

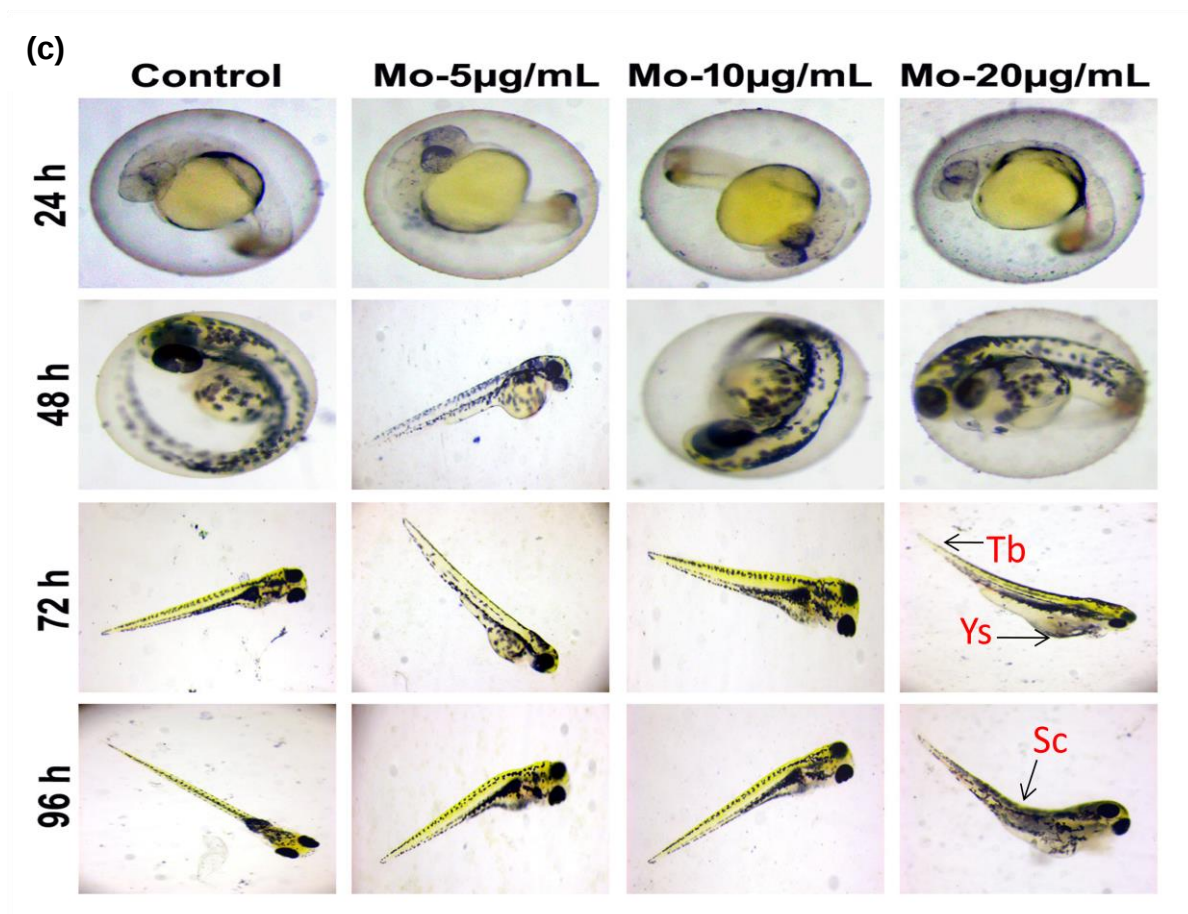
The teratogenic activity of axitinib and monacolin-x as individual and combination on embryos was assessed based on different morphological endpoints of embryos exposed to the varying concentrations of axitinib (10µg/mL, 20µg/mL and 40µg/mL), monacolin-x (5µg/mL, 10µg/mL and 20µg/mL) and axitinib+monacolin-x (5µg/mL+20µg/mL, 10µg/mL+10µg/mL and 20µg/mL+5µg/mL) was observed from 24-96 hrs. In axitinib+monacolin-x exposure, embryos at (5µg/mL+20µg/mL) concentration show pericardial edema, spinal curvature and tail bent; (20µg/mL+5µg/mL) concentration shows yolk sac development and pericardial edema; and then at (10µg/mL+10µg/mL) concentration shows only yolk sac development (**Fig 5.10a**). In axitinib individual treatment embryos at a concentration (10µg/mL) shows yolk sac development and pericardial edema; concentration of (40µg/mL) shows tail bent, spinal curvature and yolk sac development; and then at a concentration of (20µg/mL) shows yolk sac development only (**Fig 5.10b**). In monacolin-x individual treatment embryos exposed shows yolk sac development, tail bent and spinal curvature at (20µg/mL) concentration and at concentrations of (5µg/mL and 10µg/mL) shows no deformities (**Fig 5.10c**). Control embryos showed no deformities. Hence, embryos exposed to axitinib+monacolin-x combination at (10µg/mL+10µg/mL) concentration was found to be less toxic and which could be a better parameter in further exploring and characterizing the axitinib and monacolin-x combination.



**Figure 5.10 (a)** Effect of Axitinib and Monacolin-x combination on the development of zebrafish embryos in different time intervals from 24-96 hrs at various concentrations (5 $\mu$ g/mL+20 $\mu$ g/mL, 10 $\mu$ g/mL+10 $\mu$ g/mL, 20 $\mu$ g/mL+5 $\mu$ g/mL). Sc- Spinal curvature; Tb-Tail bent; Ys-Yolk sac; Pe-Pericardial edema.



**Figure 5.10 (b)** Effect of Axitinib as individual treatment on the development of zebrafish embryos in different time intervals from 24-96 hrs at various concentrations (10 $\mu$ g/mL, 20 $\mu$ g/mL, 40 $\mu$ g/mL). Sc-Spinal curvature; Tb-Tail bent; Ys-Yolk sac; Pe-Pericardial edema.

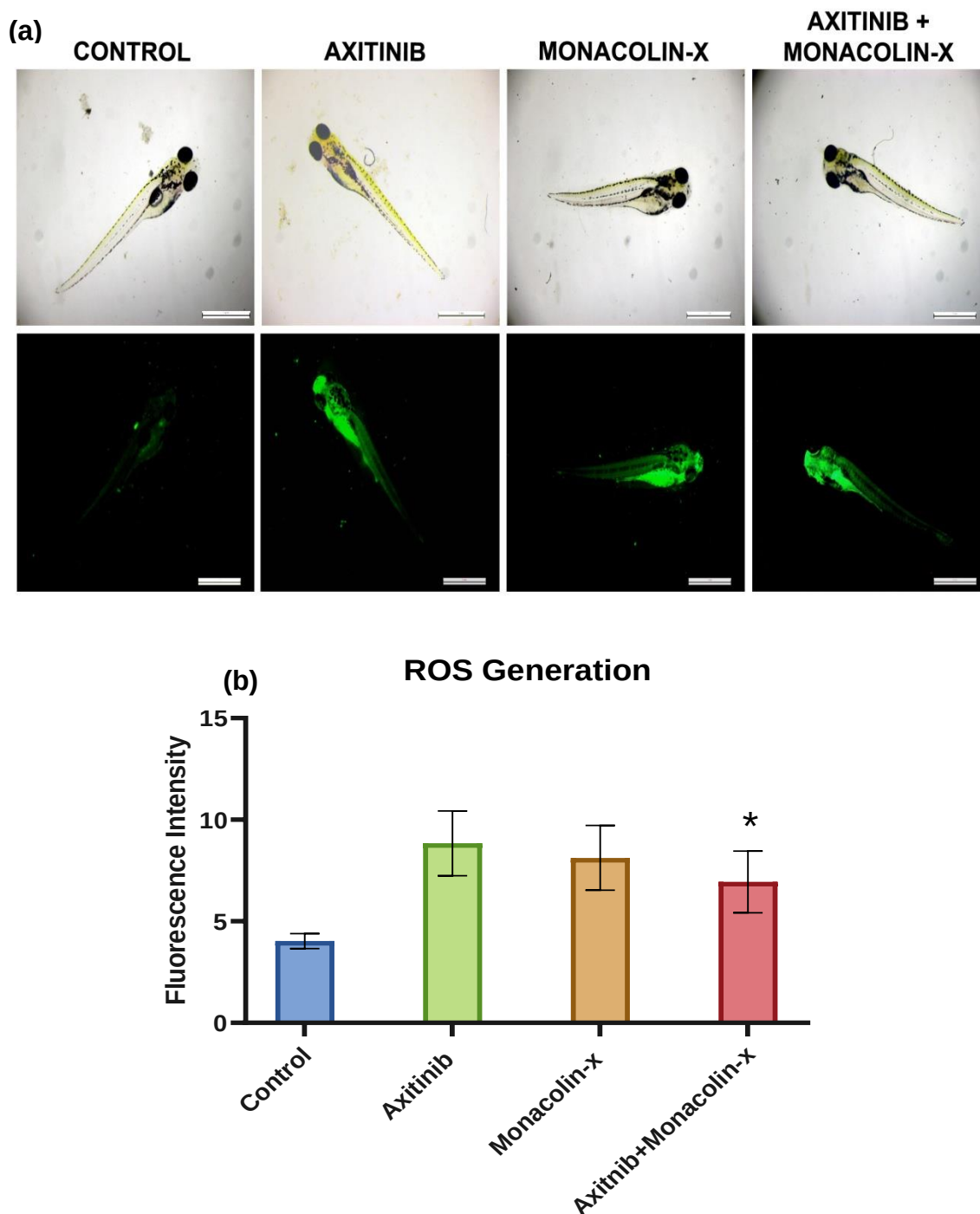


**Figure 5.10 (c)** Effect of Monacolin-x as individual treatment on the development of zebrafish embryos in different time intervals from 24-96 hrs at various concentrations (5µg/mL, 10µg/mL, 20µg/mL). Sc-Spinal curvature; Tb-Tail bent; Ys-Yolk sac.

### 5.2.3 ROS level in zebrafish larvae

DCFH-DA fluorescent staining was performed in zebrafish larvae treated with axitinib (10µg/mL), monacolin-x (10µg/mL) and axitinib+monacolin-x (10µg/mL+10µg/mL) concentrations in 96 h to evaluate in-vivo ROS inhibition efficacy. The ROS level in axitinib (10µg/mL) treatment was observed to be 8.8%, monacolin-x (10µg/mL) treatment was observed to be 8.1% and found to be almost similar. The ROS level in axitinib+monacolin-x (10µg/mL+10µg/mL) was observed to be 6.9% and in control it was observed to be 4% (**Fig 5.11a**). Zebrafish larvae exposed to axitinib+monacolin-x as combination showed significantly decreased levels of ROS generation when compared to axitinib, monacolin-x as individual and increase level of ROS when compared to control.

Hence, embryos exposed to axitinib+monacolin-x at (10 $\mu$ g/mL+10 $\mu$ g/mL) inhibits ROS generation as combination.



**Figure 5.11 ROS production in zebrafish larvae at 96 h. (a)** DCFH-DA staining on zebrafish larvae treated with axitinib (10 $\mu$ g/mL), monacolin-x (10 $\mu$ g/mL) and axitinib+monacolin-x (10 $\mu$ g/mL+10 $\mu$ g/mL) in 96 h. **(b)** Quantitative analysis of ROS generation in zebrafish larvae by Image J software. The values were expressed in mean $\pm$ SD (n=10); statistical analysis was performed using one way ANOVA

*followed by Tukey's multiple comparison test, \*indicates p value<0.05 vs. axitinib, monacolin-x and control.*

Combination regimens of two or more drugs for breast cancer were commonly used in clinical practice, and we investigated the specific effects between axitinib and monacolin-x. The aim of this study is to explore the therapeutic efficiency of axitinib and monacolin-x as a combination for treatment against human breast cancer cell line through *In vitro* and *In vivo* approach the study sought to provide comprehensive insights in pharmaceutical biotechnology which certainly coordinates with program outcomes.

The cytotoxic activity of axitinib and monacolin-x as individual and combination were assessed using MTT assay against MCF-7 and MDA-MB-231 breast cancer cells. Axitinib and monacolin-x combination showed a decrease in the number of viable cells due to an increase of cell death and/or decreased cell proliferation in a dose-dependent manner, with an IC<sub>50</sub> value 82.7µM on MCF-7 and 93.3µM on MDA-MB-231 respectively. Axitinib and monacolin-x alone also showed a decrease in cell viability on both MCF-7 and MDA-MB-231 with an IC<sub>50</sub> value of axitinib alone 27.55µM and 40.31µM then monacolin-x alone 56.14µM and 66.2µM respectively. Statistical analysis suggests that axitinib and monacolin-x combination shows significant difference compared to axitinib and monacolin-x individual treatment on MDA-MB-231 cells and no statistical significant difference in MCF-7 cells. In a previous study by (Nagabishek and Madankumar, 2019) reported that the IC<sub>50</sub> value of monacolin-x in MCF-7 and MDA-MB-231 was found to be 42.87 µM and 48.68 µM respectively. Axitinib and monacolin-x combination showed synergistic effect (CI<1) on MDA-MB-231 cells and in MCF-7 cells synergistic effect (CI<1) was observed when Fa value was <0.5 and Fa value >0.5 showed additive effect (CI=1). A study reported that axitinib combined with 5-fluorouracil (5-FU) had synergistic inhibitory effect (CI < 1) when Fa value was ≤0.8, while synergism between axitinib and DDP (CI < 1) was observed when Fa value was >0.4 in BGC-823 cells (He *et al.*, 2014). Apoptosis induction was confirmed by PI and AO/EB staining, where the IC<sub>50</sub> value was used to evaluate the apoptosis induced by axitinib and monacolin-x combination on MDA-MB-231 cells. PI staining is used to show the morphological changes under a fluorescence microscope and to distinguishing between cell death mediated by apoptosis or

necrosis and morphological observation of apoptotic cells are distinguished by cell shrinkage, chromatin condensation, membrane blebbing, DNA fragmentation and formation of small apoptotic bodies. The percentage of apoptotic nuclei was considerably increased. AO/EB staining enables to distinguish between necrotic cells, early apoptosis cells and late apoptosis cells. Axitinib and monacolin-x treatment indicated higher proportion of late apoptotic cells confirmed by AO/EB staining. Thus, axitinib and monacolin-x combination was able to induce nuclear damage and apoptosis, further supporting the role of reactive oxygen species (ROS). Generally, ROS have a dual role, either beneficial or harmful depending on their levels of accumulation and contributes to cell death here axitinib and monacolin-x combination treatment shows significantly increased levels of ROS generation compared to axitinib, monacolin-x individual treatment and control. In this study, the antioxidative property was assessed using DPPH for measuring radical scavenging activity of axitinib and monacolin-x combination. The antioxidant assay results revealed that the axitinib and monacolin-x combination exhibited a linear increase in DPPH radical scavenging activity with respect to dose dependent manner.

A diverse range of genes participate in the process of angiogenesis and the most researched pathways for angiogenesis involve vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs). However, it has been documented that various other genetic pathways also play a crucial role in angiogenesis by several factors such as hypoxia and localized inflammation tumor angiogenesis. Pro-inflammatory cytokines like interleukin IL-1 $\beta$  and IL-6 function through different mediators, including tumor necrosis factor TNF- $\alpha$ , to either enhance or suppress angiogenesis, depending on the concentration and duration of exposure to cytokines. These elements might contribute to the tumor's capacity to invade vessels and spread to other parts of the body and NF- $\kappa$ B in breast cancer cells leads to the induction of neo-vascularization angiogenesis, epithelial-mesenchymal transition (EMT), and an increase in cancer cell stemness. Gene expression analysis using qRT-PCR results revealed that axitinib and monacolin-x combination treatment led to significant downregulated the mRNA expression of genes TNF- $\alpha$ , IL-6, IL-1 $\beta$  and NF- $\kappa$ B, suggesting its role in downregulation of inflammatory and angiogenesis pathways.

Further, the toxicity study on zebrafish embryos by investigating the embryotoxic and teratogenic effects of the axitinib and monacolin-X combination revealed that the combination at (10µg/mL+10µg/mL) concentration showed no relevant changes in the survival rate, hatching rate and heart beat rate of larvae exposed up to 96 hrs and then showed only yolk sac development. Zebrafish larvae exposed to axitinib and monacolin-x combination at (10µg/mL+10µg/mL) concentration significantly inhibited ROS generation when compared to axitinib, monacolin-x individual treatment and it was found to be low toxic. A similar study by (D'Iglio *et al.*, 2023) reported that combination of Gemcitabine and paclitaxel exposure at single non-toxic concentrations affected survival and hatching rate. Additionally, exposure significantly disturbed the antioxidant defense system and increased ROS in zebrafish larvae.

Overall, the findings from the study provide therapeutic potential of axitinib and monacolin-x combination with proven statistical analysis which satisfies the student outcome for ability to develop and conduct experiments and testing hypothesis, analyze and interpret data and use scientific judgment to draw conclusions. These results suggest that this could be a better parameter in further exploring and characterizing the axitinib and monacolin-x combination as potential therapeutic application in treatment for metastatic breast cancer.

## CHAPTER 6

### SUMMARY AND CONCLUSION

In summary, Breast cancer, particularly triple-negative breast cancer (TNBC), presents a significant health challenge due to its aggressive nature and poor prognosis. Traditional monotherapy treatments have limitations, leading to relapses in many patients. Recent advancements in treatment strategies emphasize the importance of combination therapies to address the heterogeneity, tumor evolution, and drug resistance associated with breast cancer. In the present study, we demonstrate the combination therapy involving axitinib, a potent inhibitor of tyrosine kinases involved in angiogenesis which exerted potent antitumor activity in several solid tumors and monacolin-x, a marine sponge associated fungal secondary metabolite with anti-cancer properties, against breast cancer cells specifically targeting TNBC in both in vitro and in vivo to evaluate the efficacy of axitinib and monacolin-x combination. Experimental validation through MTT assay demonstrated dose-dependent cytotoxicity of the combination in MDA-MB-231 cells, indicating its potential to inhibit cell growth. Interestingly, the combination showed no significant cytotoxic effects in MCF-7 cells, suggesting a selective impact on TNBC cells and showed synergistic effects as combination. Furthermore, the combination of axitinib and monacolin-x induced apoptosis in MDA-MB-231 cells, as evidenced by the presence of apoptotic bodies observed through propidium iodide (PI) and acridine orange/ethidium bromide (AO/EB) dual staining. This apoptotic activity highlights the potential of the combination to trigger programmed cell death in TNBC cells. Axitinib and monacolin-X combination have enhanced antioxidant activity evaluated through DPPH assay and increased ROS levels through validation by DCFH-DA staining, suggesting a potential role in oxidative stress modulation within cancer cells. Through qRT-PCR analysis the relative mRNA expression of the genes involved in inflammation and angiogenesis including NF- $\kappa$ B, IL-6, TNF- $\alpha$ , and IL-1 $\beta$  was assessed. The combination of axitinib and monacolin-x downregulated the expression of these genes, indicating its potential to modulate inflammatory pathways and angiogenesis that suppress tumor-promoting signals in TNBC cells. Furthermore, investigations on the embryotoxic and teratogenic effects of the axitinib and monacolin-X combination

using zebrafish embryos revealed that the combination at specific concentrations exhibited low toxicity and did not cause deformities in the zebrafish embryos. Additionally, the combination significantly inhibited ROS levels in the zebrafish embryos, suggesting a favorable safety profile for potential future applications.

In conclusion, combination of axitinib and monacolin-x exerts potent efficacy and anti-cancer activity against breast cancer cell line MDA-MB-231 in vitro and in vivo studies on zebrafish embryos confirmed their low toxicity in combination suggesting potential therapeutic application in treatment for metastatic breast cancer. However, further research is needed to fully evaluate the safety and efficacy of this combination but early results are promising and warrant continued investigation. If successful, this approach could significantly improve the outlook for patients with breast cancer, offering a more targeted and comprehensive treatment option.

## REFERENCES

- Al-Hilli, Z., Wilkerson, A., 2021. Breast Surgery: Management of Postoperative Complications Following Operations for Breast Cancer. *Surg Clin North Am* 101, 845-863.
- Anand, P., Kunnumakkara, A.B., Sundaram, C., Harikumar, K.B., Tharakan, S.T., Lai, O.S., Sung, B., Aggarwal, B.B., 2008. Cancer is a preventable disease that requires major lifestyle changes. *Pharm Res* 25, 2097-2116.
- Ansari, M.J., Bokov, D., Markov, A., Jalil, A.T., Shalaby, M.N., Suksatan, W., Chupradit, S., Al-Ghamdi, H.S., Shomali, N., Zamani, A., Mohammadi, A., Dadashpour, M., 2022. Cancer combination therapies by angiogenesis inhibitors; a comprehensive review. *Cell Commun Signal* 20, 49.
- Arnold, M., Morgan, E., Rungay, H., Mafra, A., Singh, D., Laversanne, M., Vignat, J., Gralow, J.R., Cardoso, F., Siesling, S., Soerjomataram, I., 2022. Current and future burden of breast cancer: Global statistics for 2020 and 2040. *Breast* 66, 15-23.
- Baselga, J., Albanell, J., Ruiz, A., Lluch, A., Gascon, P., Guillem, V., Gonzalez, S., Sauleda, S., Marimon, I., Tabernero, J.M., Koehler, M.T., Rojo, F., 2005. Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. *J Clin Oncol* 23, 5323-5333.
- Bayat Mokhtari, R., Homayouni, T.S., Baluch, N., Morgatskaya, E., Kumar, S., Das, B., Yeger, H., 2017. Combination therapy in combating cancer. *Oncotarget* 8, 38022-38043.
- Bigda, J., Okrój, M., 2002. The role of tumor necrosis factor (TNF) in angiogenesis. *Contemporary Oncology/Współczesna Onkologia* 6, 57-59.
- Burguin, A., Diorio, C., Durocher, F., 2021. Breast Cancer Treatments: Updates and New Challenges. *J Pers Med* 11.

Cai, X., Cao, C., Li, J., Chen, F., Zhang, S., Liu, B., Zhang, W., Zhang, X., Ye, L., 2017. Inflammatory factor TNF- $\alpha$  promotes the growth of breast cancer via the positive feedback loop of TNFR1/NF- $\kappa$ B (and/or p38)/p-STAT3/HBXIP/TNFR1. *Oncotarget* 8, 58338-58352.

Correia, A.S., Gärtner, F., Vale, N., 2021. Drug combination and repurposing for cancer therapy: the example of breast cancer. *Heliyon* 7, e05948.

D'Iglio, C., Famulari, S., Capparucci, F., Gervasi, C., Cuzzocrea, S., Spano, N., Di Paola, D., 2023. Toxic Effects of Gemcitabine and Paclitaxel Combination: Chemotherapy Drugs Exposure in Zebrafish. *Toxics* 11.

Dicitore, A., Gaudenzi, G., Carra, S., Cantone, M.C., Oldani, M., Saronni, D., Borghi, M.O., Grotteschi, J., Persani, L., Vitale, G., 2023. Antitumor Activity of Axitinib in Lung Carcinoids: A Preclinical Study. *Cancers (Basel)* 15.

Du Four, S., Maenhout, S.K., De Pierre, K., Renmans, D., Niclou, S.P., Thielemans, K., Neyns, B., Aerts, J.L., 2015. Axitinib increases the infiltration of immune cells and reduces the suppressive capacity of monocytic MDSCs in an intracranial mouse melanoma model. *Oncoimmunology* 4, e998107.

Duarte, D., Cardoso, A., Vale, N., 2021. Synergistic Growth Inhibition of HT-29 Colon and MCF-7 Breast Cancer Cells with Simultaneous and Sequential Combinations of Antineoplastics and CNS Drugs. *Int J Mol Sci* 22.

Endo, A., Hasumi, K., Nakamura, T., Kunishima, M., Masuda, M., 1985. Dihydromonacolin L and monacolin X, new metabolites which inhibit cholesterol biosynthesis. *J Antibiot (Tokyo)* 38, 321-327.

Eswaraka, J., Giddabasappa, A., Han, G., Lalwani, K., Eisele, K., Feng, Z., Affolter, T., Christensen, J., Li, G., 2014. Axitinib and crizotinib combination therapy inhibits bone loss in a mouse model of castration resistant prostate cancer. *BMC Cancer* 14, 742.

Fruehauf, J., Lutzky, J., McDermott, D., Brown, C.K., Meric, J.B., Rosbrook, B., Shalinsky, D.R., Liau, K.F., Niethammer, A.G., Kim, S., Rixe, O., 2011.

Multicenter, phase II study of axitinib, a selective second-generation inhibitor of vascular endothelial growth factor receptors 1, 2, and 3, in patients with metastatic melanoma. *Clin Cancer Res* 17, 7462-7469.

Garcia-Parra, J., Dalmases, A., Morancho, B., Arpi, O., Menendez, S., Sabbaghi, M., Zazo, S., Chamizo, C., Madoz, J., Eroles, P., Servitja, S., Tusquets, I., Yelamos, J., Lluch, A., Arribas, J., Rojo, F., Rovira, A., Albanell, J., 2014. Poly (ADP-ribose) polymerase inhibition enhances trastuzumab antitumour activity in HER2 overexpressing breast cancer. *Eur J Cancer* 50, 2725-2734.

Hashemi, F., Zarrabi, A., Zabolian, A., Saleki, H., Farahani, M.V., Sharifzadeh, S.O., Ghahremaniyeh, Z., Bejandi, A.K., Hushmandi, K., Ashrafizadeh, M., Khan, H., 2021. Novel Strategy in Breast Cancer Therapy: Revealing The Bright Side of Ginsenosides. *Curr Mol Pharmacol* 14, 1093-1111.

He, Q., Gao, J., Ge, S., Wang, T., Li, Y., Peng, Z., Li, Y., Shen, L., 2014. Axitinib alone or in combination with chemotherapeutic drugs exerts potent antitumor activity against human gastric cancer cells in vitro and in vivo. *J Cancer Res Clin Oncol* 140, 1575-1583.

Herzog, S.K., Fuqua, S.A.W., 2022. ESR1 mutations and therapeutic resistance in metastatic breast cancer: progress and remaining challenges. *Br J Cancer* 126, 174-186.

Hu-Lowe, D.D., Zou, H.Y., Grazzini, M.L., Hallin, M.E., Wickman, G.R., Amundson, K., Chen, J.H., Rewolinski, D.A., Yamazaki, S., Wu, E.Y., McTigue, M.A., Murray, B.W., Kania, R.S., O'Connor, P., Shalinsky, D.R., Bender, S.L., 2008. Nonclinical antiangiogenesis and antitumor activities of axitinib (AG-013736), an oral, potent, and selective inhibitor of vascular endothelial growth factor receptor tyrosine kinases 1, 2, 3. *Clin Cancer Res* 14, 7272-7283.

Hwang, J.T., Ha, J., Park, O.J., 2005. Combination of 5-fluorouracil and genistein induces apoptosis synergistically in chemo-resistant cancer cells through the modulation of AMPK and COX-2 signaling pathways. *Biochem Biophys Res Commun* 332, 433-440.

Kashyap, D., Pal, D., Sharma, R., Garg, V.K., Goel, N., Koundal, D., Zaguia, A., Koundal, S., Belay, A., 2022. Global Increase in Breast Cancer Incidence: Risk Factors and Preventive Measures. *Biomed Res Int* 2022, 9605439.

Kerr, A.J., Dodwell, D., McGale, P., Holt, F., Duane, F., Mannu, G., Darby, S.C., Taylor, C.W., 2022. Adjuvant and neoadjuvant breast cancer treatments: A systematic review of their effects on mortality. *Cancer Treat Rev* 105, 102375.

Lakshmaiah, K.C., Das, U., Suresh, T.M., Lokanatha, D., Babu, G.K., Jacob, L.A., Babu, S., 2014. A study of triple negative breast cancer at a tertiary cancer care center in southern India. *Ann Med Health Sci Res* 4, 933-937.

Lee, H.G., Lim, G.H., An, J.H., Park, S.M., Seo, K.W., Youn, H.Y., 2024. In vitro evaluation of the antitumor activity of axitinib in canine mammary gland tumor cell lines. *J Vet Sci* 25, e1.

Li, C.J., Tzeng, Y.T., Chiu, Y.H., Lin, H.Y., Hou, M.F., Chu, P.Y., 2021. Pathogenesis and Potential Therapeutic Targets for Triple-Negative Breast Cancer. *Cancers (Basel)* 13.

Luo, C., Wang, P., He, S., Zhu, J., Shi, Y., Wang, J., 2022. Progress and Prospect of Immunotherapy for Triple-Negative Breast Cancer. *Front Oncol* 12, 919072.

Ma, Y.H., Wang, S.Y., Ren, Y.P., Li, J., Guo, T.J., Lu, W., Zhou, T.Y., 2019. Antitumor effect of axitinib combined with dopamine and PK-PD modeling in the treatment of human breast cancer xenograft. *Acta Pharmacol Sin* 40, 243-256.

Manore, S.G., Doheny, D.L., Wong, G.L., Lo, H.W., 2022. IL-6/JAK/STAT3 Signaling in Breast Cancer Metastasis: Biology and Treatment. *Front Oncol* 12, 866014.

Mansur, A., Song, P.N., Lu, Y., Burns, A.C., Sligh, L., Yang, E.S., Sorace, A.G., 2023. Combination Therapy with Trastuzumab and Niraparib: Quantifying Early Proliferative Alterations in HER2+ Breast Cancer Models. *Biomedicines* 11.

Martin, L.P., Kozloff, M.F., Herbst, R.S., Samuel, T.A., Kim, S., Rosbrook, B., Tortorici, M., Chen, Y., Tarazi, J., Olszanski, A.J., Rado, T., Starr, A., Cohen, R.B., 2012. Phase I study of axitinib combined with paclitaxel, docetaxel or capecitabine in patients with advanced solid tumours. *Br J Cancer* 107, 1268-1276.

Masjedi, A., Hashemi, V., Hojjat-Farsangi, M., Ghalamfarsa, G., Azizi, G., Yousefi, M., Jadidi-Niaragh, F., 2018. The significant role of interleukin-6 and its signaling pathway in the immunopathogenesis and treatment of breast cancer. *Biomed Pharmacother* 108, 1415-1424.

Mehrotra, R., Yadav, K., 2022. Breast cancer in India: Present scenario and the challenges ahead. *World J Clin Oncol* 13, 209-218.

Meng, J., Dai, B., Fang, B., Bekele, B.N., Bornmann, W.G., Sun, D., Peng, Z., Herbst, R.S., Papadimitrakopoulou, V., Minna, J.D., Peyton, M., Roth, J.A., 2010. Combination treatment with MEK and AKT inhibitors is more effective than each drug alone in human non-small cell lung cancer in vitro and in vivo. *PLoS One* 5, e14124.

Mongiardi, M.P., Radice, G., Piras, M., Stagni, V., Pacioni, S., Re, A., Putti, S., Ferre, F., Farsetti, A., Pallini, R., Barila, D., Levi, A., Falchetti, M.L., 2019. Axitinib exposure triggers endothelial cells senescence through ROS accumulation and ATM activation. *Oncogene* 38, 5413-5424.

Montagna, E., Canello, G., Bagnardi, V., Pastrello, D., Dellapasqua, S., Perri, G., Viale, G., Veronesi, P., Luini, A., Intra, M., Calleri, A., Rampinelli, C., Goldhirsch, A., Bertolini, F., Colleoni, M., 2012. Metronomic Chemotherapy Combined With Bevacizumab and Erlotinib in Patients With Metastatic HER2-Negative Breast Cancer: Clinical and Biological Activity. *Clinical Breast Cancer* 12, 207-214.

Motzer, R.J., Penkov, K., Haanen, J., Rini, B., Albiges, L., Campbell, M.T., Venugopal, B., Kollmannsberger, C., Negrier, S., Uemura, M., Lee, J.L., Vasiliev, A., Miller, W.H., Jr., Gurney, H., Schmidinger, M., Larkin, J., Atkins, M.B., Bedke, J., Alekseev, B., Wang, J., Mariani, M., Robbins, P.B., Chudnovsky, A., Fowst, C., Hariharan, S., Huang, B., di Pietro, A., Choueiri, T.K., 2019. Avelumab plus

Axitinib versus Sunitinib for Advanced Renal-Cell Carcinoma. *N Engl J Med* 380, 1103-1115.

Nagabhishek, S.N., Madan Kumar, A., B, S., Balakrishnan, A., Katakia, Y.T., Chatterjee, S., Nagasundaram, N., 2019. A marine sponge associated fungal metabolite monacolin X suppresses angiogenesis by down regulating VEGFR2 signaling. *RSC Adv* 9, 26646-26667.

Nagabhishek, S.N., Madankumar, A., 2019. A novel apoptosis-inducing metabolite isolated from marine sponge symbiont *Monascus* sp. NMK7 attenuates cell proliferation, migration and ROS stress-mediated apoptosis in breast cancer cells. *RSC Advances* 9, 5878-5890.

Nagel, R., 2002. DarT: The embryo test with the Zebrafish *Danio rerio*--a general model in ecotoxicology and toxicology. *ALTEX* 19 Suppl 1, 38-48.

Ngamcherdtrakul, W., Yantasee, W., 2019. siRNA therapeutics for breast cancer: recent efforts in targeting metastasis, drug resistance, and immune evasion. *Transl Res* 214, 105-120.

Nisar, M.A., Zheng, Q., Saleem, M.Z., Ahmmed, B., Ramzan, M.N., Ud Din, S.R., Tahir, N., Liu, S., Yan, Q., 2021. IL-1 $\beta$  Promotes Vasculogenic Mimicry of Breast Cancer Cells Through p38/MAPK and PI3K/Akt Signaling Pathways. *Front Oncol* 11, 618839.

Nowsheen, S., Cooper, T., Stanley, J.A., Yang, E.S., 2012. Synthetic lethal interactions between EGFR and PARP inhibition in human triple negative breast cancer cells. *PLoS One* 7, e46614.

Orrantia-Borunda, E., Anchondo-Nunez, P., Acuna-Aguilar, L.E., Gomez-Valles, F.O., Ramirez-Valdespino, C.A., 2022. Subtypes of Breast Cancer, in: Mayrovitz, H.N. (Ed.), *Breast Cancer*, Brisbane (AU).

Paik, E.S., Kim, T.H., Cho, Y.J., Ryu, J., Choi, J.J., Lee, Y.Y., Kim, T.J., Choi, C.H., Kim, W.Y., Sa, J.K., Lee, J.K., Kim, B.G., Bae, D.S., Han, H.D., Ahn, H.J.,

Lee, J.W., 2020. Preclinical assessment of the VEGFR inhibitor axitinib as a therapeutic agent for epithelial ovarian cancer. *Sci Rep* 10, 4904.

Paul, M.K., Mukhopadhyay, A.K., 2004. Tyrosine kinase - Role and significance in Cancer. *Int J Med Sci* 1, 101-115.

Pavitra, E., Kancharla, J., Gupta, V.K., Prasad, K., Sung, J.Y., Kim, J., Tej, M.B., Choi, R., Lee, J.H., Han, Y.K., Raju, G.S.R., Bhaskar, L., Huh, Y.S., 2023. The role of NF-kappaB in breast cancer initiation, growth, metastasis, and resistance to chemotherapy. *Biomed Pharmacother* 163, 114822.

Pescia, C., Guerini-Rocco, E., Viale, G., Fusco, N., 2023. Advances in Early Breast Cancer Risk Profiling: From Histopathology to Molecular Technologies. *Cancers (Basel)* 15.

Poller, B., Iusuf, D., Sparidans, R.W., Wagenaar, E., Beijnen, J.H., Schinkel, A.H., 2011. Differential impact of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) on axitinib brain accumulation and oral plasma pharmacokinetics. *Drug Metab Dispos* 39, 729-735.

Quinn, B.A., Dash, R., Sarkar, S., Azab, B., Bhoopathi, P., Das, S.K., Emdad, L., Wei, J., Pellecchia, M., Sarkar, D., Fisher, P.B., 2015. Pancreatic Cancer Combination Therapy Using a BH3 Mimetic and a Synthetic Tetracycline. *Cancer Res* 75, 2305-2315.

Rastogi, R.P., Singh, S.P., Hader, D.P., Sinha, R.P., 2010. Detection of reactive oxygen species (ROS) by the oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate in the cyanobacterium *Anabaena variabilis* PCC 7937. *Biochem Biophys Res Commun* 397, 603-607.

Rini, B., Rixe, O., Bukowski, R., Michaelson, M.D., Wilding, G., Hudes, G., Bolte, O., Steinfeldt, H., Reich, S.D., Motzer, R., 2005. AG-013736, a multi-target tyrosine kinase receptor inhibitor, demonstrates anti-tumor activity in a Phase 2 study of cytokine-refractory, metastatic renal cell cancer (RCC). *Journal of Clinical Oncology* 23, 4509-4509.

Robado de Lope, L., Sanchez-Herrero, E., Serna-Blasco, R., Provencio, M., Romero, A., 2023. Cancer as an infective disease: the role of EVs in tumorigenesis. *Mol Oncol* 17, 390-406.

Rugo, H.S., Stopeck, A.T., Joy, A.A., Chan, S., Verma, S., Lluch, A., Liau, K.F., Kim, S., Bycott, P., Rosbrook, B., Bair, A.H., Soulieres, D., 2011. Randomized, placebo-controlled, double-blind, phase II study of axitinib plus docetaxel versus docetaxel plus placebo in patients with metastatic breast cancer. *J Clin Oncol* 29, 2459-2465.

Sainson, R.C., Johnston, D.A., Chu, H.C., Holderfield, M.T., Nakatsu, M.N., Crampton, S.P., Davis, J., Conn, E., Hughes, C.C., 2008. TNF primes endothelial cells for angiogenic sprouting by inducing a tip cell phenotype. *Blood* 111, 4997-5007.

Schwartzberg, L.S., Franco, S.X., Florance, A., O'Rourke, L., Maltzman, J., Johnston, S., 2010. Lapatinib plus Letrozole as First-Line Therapy for HER-2+ Hormone Receptor–Positive Metastatic Breast Cancer. *The Oncologist* 15, 122-129.

Sheikh-Hosseini, M., Larijani, B., Gholipour Kakroodi, Z., Shokoohi, M., Moarefzadeh, M., Sayahpour, F.A., Goodarzi, P., Arjmand, B., 2021. Gene Therapy as an Emerging Therapeutic Approach to Breast Cancer: New Developments and Challenges. *Hum Gene Ther* 32, 1330-1345.

Shostak, K., Chariot, A., 2011. NF-kappaB, stem cells and breast cancer: the links get stronger. *Breast Cancer Res* 13, 214.

Shyam Sunder, S., Sharma, U.C., Pokharel, S., 2023. Adverse effects of tyrosine kinase inhibitors in cancer therapy: pathophysiology, mechanisms and clinical management. *Signal Transduct Target Ther* 8, 262.

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F., 2021. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 71, 209-249.

Takabatake, D., Fujita, T., Shien, T., Kawasaki, K., Taira, N., Yoshitomi, S., Takahashi, H., Ishibe, Y., Ogasawara, Y., Doihara, H., 2007. Tumor inhibitory effect of gefitinib (ZD1839, Iressa) and taxane combination therapy in EGFR-overexpressing breast cancer cell lines (MCF7/ADR, MDA-MB-231). *Int J Cancer* 120, 181-188.

Trayes, K.P., Cokenakes, S.E.H., 2021. Breast Cancer Treatment. *Am Fam Physician* 104, 171-178.

Truong, L., Harper, S.L., Tanguay, R.L., 2011. Evaluation of embryotoxicity using the zebrafish model. *Methods Mol Biol* 691, 271-279.

Turner, K.M., Yeo, S.K., Holm, T.M., Shaughnessy, E., Guan, J.L., 2021. Heterogeneity within molecular subtypes of breast cancer. *Am J Physiol Cell Physiol* 321, C343-C354.

Wang, W., Nag, S.A., Zhang, R., 2015. Targeting the NFkappaB signaling pathways for breast cancer prevention and therapy. *Curr Med Chem* 22, 264-289.

Wang, Y., Minden, A., 2022. Current Molecular Combination Therapies Used for the Treatment of Breast Cancer. *Int J Mol Sci* 23.

Wilson, B.E., Shen, Q., Cescon, D.W., Reedijk, M., 2023. Exploring immune interactions in triple negative breast cancer: IL-1beta inhibition and its therapeutic potential. *Front Genet* 14, 1086163.

Yang, X., Wu, D., Yuan, S., 2020. Tyrosine Kinase Inhibitors in the Combination Therapy of HER2 Positive Breast Cancer. *Technol Cancer Res Treat* 19, 1533033820962140.

Yao, M., Yuan, B., Wang, X., Sato, A., Sakuma, K., Kaneko, K., Komuro, H., Okazaki, A., Hayashi, H., Toyoda, H., Pei, X., Hu, X., Hirano, T., Takagi, N., 2017. Synergistic cytotoxic effects of arsenite and tetrandrine in human breast cancer cell line MCF-7. *Int J Oncol* 51, 587-598.

Yuan, B., Yao, M., Wang, X., Sato, A., Okazaki, A., Komuro, H., Hayashi, H., Toyoda, H., Pei, X., Hu, X., Hirano, T., Takagi, N., 2018. Antitumor activity of

arsenite in combination with tetrandrine against human breast cancer cell line MDA-MB-231 in vitro and in vivo. *Cancer Cell Int* 18, 113.

Zhang, N., Dai, L., Qi, Y., Di, W., Xia, P., 2013. Combination of FTY720 with cisplatin exhibits antagonistic effects in ovarian cancer cells: role of autophagy. *Int J Oncol* 42, 2053-2059.

Zhang, N., Fu, J.N., Chou, T.C., 2016a. Synergistic combination of microtubule targeting anticancer fludelone with cytoprotective panaxytriol derived from panax ginseng against MX-1 cells in vitro: experimental design and data analysis using the combination index method. *Am J Cancer Res* 6, 97-104.

Zhang, Y., Yang, C., Wang, W., Liu, J., Liu, Q., Huang, F., Chu, L., Gao, H., Li, C., Kong, D., Liu, Q., Liu, J., 2016b. Co-delivery of doxorubicin and curcumin by pH-sensitive prodrug nanoparticle for combination therapy of cancer. *Scientific Reports* 6, 21225.

## ANNEXURE

### Review paper Manuscript submitted to the journal

Kathir Vel <kathir06301@gmail.com>

---

**Please verify your contribution to Recent advancements in small interfering RNA (siRNA) based therapeutic approach on breast cancer**  
1 message

---

**The European Journal of Pharmacology** <em@editorialmanager.com> Sat, Mar 2, 2024 at 3:53 PM  
Reply-To: The European Journal of Pharmacology <support@elsevier.com>  
To: Kathirvel Kalaimani <kathir06301@gmail.com>

\*This is an automated message.\*

Journal: European Journal of Pharmacology  
Title: Recent advancements in small interfering RNA (siRNA) based therapeutic approach on breast cancer  
Corresponding Author: Dr. Madan Kumar Arumugam  
Co-Authors: Kathirvel Kalaimani, BTech; Shana Balachandran, MSc; Lokesh Kumar Boopathy, PhD; Anitha Roy, PhD; Rakhee Rathnam Kalani Kandy, PhD  
Manuscript Number: **EJP-69827**

Dear Kathirvel Kalaimani,

The corresponding author Dr. Madan Kumar Arumugam has listed you as a contributing author of the following submission via Elsevier's online submission system for European Journal of Pharmacology.

Submission Title: Recent advancements in small interfering RNA (siRNA) based therapeutic approach on breast cancer

Elsevier asks all authors to verify their co-authorship by confirming agreement to publish this article if it is accepted for publication.

Please read the following statement and confirm your agreement by clicking on this link: [Yes, I am affiliated.](#)

I irrevocably authorize and grant my full consent to the corresponding author of the manuscript to: (1) enter into an exclusive publishing agreement with Elsevier on my behalf (or, if the article is to be published under a CC BY license, a non-exclusive publishing agreement), in the relevant form set out at [www.elsevier.com/copyright](http://www.elsevier.com/copyright); and (2) unless I am a US government employee, to transfer my copyright or grant an exclusive license of rights (or for CC BY articles a non-exclusive license of rights) to Elsevier as part of that publishing agreement, effective on acceptance of the article for publication. If the article is a work made for hire, I am authorized to confirm this on behalf of my employer. I agree that the copyright status selected by the corresponding author for the article if it is accepted for publication shall apply and that this agreement is subject to the governing law of the country in which the journal owner is located.

If you did not co-author this submission, please contact the corresponding author directly at [madankumarbio@gmail.com](mailto:madankumarbio@gmail.com); [madankumar@sathyabamauniversity.ac.in](mailto:madankumar@sathyabamauniversity.ac.in).

Thank you,  
European Journal of Pharmacology

## GATE SCORECARD

Your GATE 2024 Result [BT]

Name

KATHIRVEL K

Registration Number

BT24S57403136

Gender

Male

Parent's/Guardian's name

KALAIMANI R

Date of Birth (YYYY-MM-DD)

2002-11-06

Examination Paper

Biotechnology (BT)



Photograph

A handwritten signature in blue ink that reads "Kathirvel K" on a grey background.

Signature

Marks out of 100<sup>#</sup>

20.67

Qualifying Marks<sup>##</sup>

38.9

35

25.9

General OBC-NCL/EWS SC/ST/PwD

<sup>#</sup>Normalized marks in case of multisession papers (CE and CS).

<sup>##</sup>A candidate is considered qualified if the marks secured are greater than or equal to the qualifying marks mentioned for the category, for which a valid category certificate, if applicable, must be produced along with the Score Card.

[FAQ for GATE Score](#)

CLOSE

PLAGIARISM REPORT



### Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

|                    |                                |
|--------------------|--------------------------------|
| Submission author: | kathir vel                     |
| Assignment title:  | TechBee Premium                |
| Submission title:  | vel                            |
| File name:         | PROJECT_THESIS.docx            |
| File size:         | 4MB                            |
| Page count:        | 80                             |
| Word count:        | 17,413                         |
| Character count:   | 120,165                        |
| Submission date:   | 27-Mar-2024 06:18PM (UTC-0700) |
| Submission ID:     | 2230032262                     |



Copyright 2024 Turnitin. All rights reserved.

vel

ORIGINALITY REPORT

12%  
SIMILARITY INDEX

8%  
INTERNET SOURCES

8%  
PUBLICATIONS

2%  
STUDENT PAPERS

PRIMARY SOURCES

1

www.ncbi.nlm.nih.gov

Internet Source

3%

2

sci-hub.se

Internet Source

1%

3

worldwidescience.org

Internet Source

1%

4

link.springer.com

Internet Source

1%

5

hal.archives-ouvertes.fr

Internet Source

1%

6

www.hindawi.com

Internet Source

1%