

ANTICANCER EFFECT OF MONACOLIN X AGAINST HUMAN LIVER CANCER CELL LINE

*Submitted in partial fulfilment of the requirements for the award of Master of
Science in Medical Biotechnology and Clinical Research*

By

BHUVANESHWARI J

Reg No: 42101003



**CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES
INTERNATIONAL RESEARCH CENTRE**

SATHYABAMA

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

Accredited with Grade "A++" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119

APRIL - 2024



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

ACCREDITED WITH GRADE "A++" BY NAAC
CATEGORY - 1 UNIVERSITY BY UGC

CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ms. BHUVANESHWARI J (42101003)** who carried out Project entitled "ANTICANCER EFFECT OF MONACOLIN X AGAINST HUMAN LIVER CANCER CELL LINE" under the supervision of **Dr. A. Madan Kumar, Associate Professor (Research)** from September 2023 to April 2024.

PROJECT GUIDE

Dr. A. MADAN KUMAR

HEAD OF THE DEPARTMENT

Dr. A. DAYANANDAN

Submitted for Viva-voce Examination held on 18/04/2024

Internal Examiner

External Examiner



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

ACCREDITED WITH GRADE "A++" BY NAAC
CATEGORY - 1 UNIVERSITY BY UGC

DECLARATION

I, **Ms. BHUVANESHWARI J (42101003)** hereby declare that the Project Report entitled "**ANTICANCER EFFECT OF MONACOLIN X AGAINST HUMAN LIVER CANCER CELL LINE**" done by me under the guidance of **Dr. A. Madan Kumar Associate Professor (Research)** at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

DATE: 18/04/2024

PLACE: Chennai

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

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

I convey my thanks to **Dr. A. DAYANANDAN Professor (Research)**, Head of the Department, Centre for Molecular and Nanomedical sciences for providing me necessary support and details at the right time during the progressive reviews. I would also like to express my sincere and deep sense of gratitude for his constant support and encouragement during the advancement of my project work.

I am extremely grateful to **Dr. A. MADAN KUMAR Associate Professor (Research)** my project guide for his valuable guidance, suggestions, and constant encouragement paved the way for the successful completion of my project work.

I am extremely thankful to **Dr. JOHNSON RETNARAJ SAMUEL Associate Professor (Research)**, **Dr. JACKSON DURAIRAJ Associate Professor (Research)** and **Dr. BERYL VEDHA Assistant Professor (Research)** for their valuable suggestions and support for the successful completion of my project work.

I wish to express my sincere thanks to all Research Scholars Teaching and Non-teaching staff members of the Centre for Molecular and Nanomedical sciences who were helpful in many ways for the completion of the project.

I thank my parents and family members for their continuous love and support for the completion of my project and master degree.

Bhuvaneshwari J

ABSTRACT

Liver cancer, particularly hepatocellular carcinoma (HCC), poses a significant global health burden, with limited effective treatment options. Monacolin-x, a natural compound derived from marine sponge has garnered attention for its potential anticancer properties. This study aimed to investigate the anticancer effects of Monacolin-x against a human liver cancer 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 monacolin-x. Furthermore, embryotoxic levels in zebrafish embryo were investigated. The MTT results revealed dose-dependent cytotoxicity in HepG2 cells with an IC₅₀ value of 75µM respectively and showed synergistic effect. Monacolin-x induced apoptosis, it was evident by PI and AO/EB staining, showed enhanced antioxidant activity, increased intracellular ROS levels and downregulated IL-6, TNF-α, IL-1β, Bax and BCL-2 mRNA expressions through qRT-PCR analysis. The embryotoxic studies on zebrafish embryos revealed the monacolin-x was found to be less toxic with no such deformities. Therefore, monacolin-x has potent anti-cancer activity against HepG2 cell line and further studies are needed to explore the synergistic effects of these studies for clinical implications in liver cancer.

Keywords: Liver cancer, Monacolin-x, Hepatocellular carcinoma, Anticancer, HepG2, Apoptosis.

TABLE OF CONTENTS

CHAPTER NO	TITLE	PAGE NUMBER
	LIST OF ABBREVIATIONS	
	LIST OF FIGURES	
	LIST OF TABLES	
1.	INTRODUCTION	
	1.1 CANCER	1
	1.1.2 CANCER CELL CHARACTERISTICS	3
	1.1.3 CANCER DEVELOPMENT	7
	1.1.4 CANCER CELL METABOLISM	8
	1.1.5 FUNCTION OF CANCER	10
	1.1.6 INCIDENCE	11
	1.1.7 TREATMENT OF CANCER	14
	1.1.8 CHEMOTHERAPY TREATMENT OF CANCER	15
	1.2 LIVER ANATOMY AND PHYSIOLOGY	16
	1.3 LIVER CANCER	17
	1.3.1 LIVER METABOLISM	18
	1.3.2 STATISTICS	21
	1.3.3 STAGES OF LIVER CANCER	22
	1.3.4 SYMPTOMS OF LIVER CANCER	23

1.3.5 RISK FACTOR	24
1.4 TREATMENT OF LIVER CANCER	25
1.5 CHEMOTHERAPY	26
1.6 MONOCOLIN	27
1.7 MONACOLIN X	27
1.7.1 ANTICANCER MECHANISM	28
1.7.2 PRECLINICAL AND CLINICAL EVIDENCE	29
1.7.3 CHALLENGES AND FUTURE DIRECTION	30
1.7.4 CHEMICAL PROPERTIES OF MONACOLIN X	31
1.7.5 PHYSICAL PROPERTIES OF MONACOLIN X	32
2. LITERATURE SURVEY	
2.1 CANCER	33
2.2 BREAST CANCER	34
2.3 LIVER CANCER	35
2.3.1 EPIDEMIOLOGY	36
2.4 MONACOLIN	37
2.5 MONACOLIN X	37
2.5.1 ROLE OF MONACOLIN X IN CANCER	38
2.6 ROLE OF IL-6 IN LIVER CANCER	38
2.7 ROLE OF IL-1 β IN LIVER CANCER	39

2.8	ROLE OF TNF- α IN LIVER CANCER	39
2.9	ROLE OF BAX AND BCL-2 IN LIVER CANCER	40
3.	AIM AND OBJECTIVES	
3.1	AIM	41
3.2	SCOPE	41
4.	MATERIALS AND METHODS	
4.1	CHEMICALS AND REAGENTS	42
4.2	INVITRO CELL LINE STUDIES	42
4.2.1	CELL LINE MAINTENANCE	43
4.2.2	EXPERIMENTAL DESIGN	44
4.2.3	DRUG PREPARATION	44
4.2.4	CYTOTOXICITY ASSAY	45
4.2.5	NECROSIS ASSAY BY PI STAINING	46
4.2.6	APOPTOSIS ASSAY	46
4.2.7	ROS STAINING	47
4.2.8	DPPH STAINING	47
4.2.9	RNA ISOLATION	48
4.2.10	RNA QUANTIFICATION	49
4.2.11	CDNA CONVERSION	49
4.2.12	PRIMER DESIGNING	50
4.2.13	RT-PCR ANALYSIS	51

4.3 TOXICOLOGY STUDIES	51
4.3.1 INVIVO DEVELOPMENTAL TOXIC LEVEL OF ZEBRAFISH USING MONACOLIN X	52
4.3.2 FISH MAINTENANCE	52
4.3.3 EXPERIMENTAL DESIGN	52
4.3.4 TREATMENT OF ZEBRAFISH EMBRYO WITH MONACOLIN X	53
4.3.5 DEVELOPMENTAL DEFORMITIES	54
4.3.6 HEART RATE	54
4.3.7 HATCHABILITY	54
4.3.8 MORTALITY	55
5. RESULTS AND DISCUSSION	
5.1 INVITRO ANTICANCER STUDIES	57
5.1.1 EXPERIMENTAL DESIGN	57
5.1.2 DRUG PREPARATION	57
5.1.3 CYTOTOXICITY ASSAY	58
5.1.4 NECROSIS ASSAY	59
5.1.5 APOPTOSIS ASSAY	61
5.1.6 ROS STAINING	63
5.1.7 DPPH FREE RADICAL SCAVENGING ASSAY	64
5.1.8 GENE EXPRESSION ANALYSIS	65
5.2 INVIVO STUDIES	70

5.2.1 EXPERIMENTAL DESIGN	70
5.2.2 EMBRYOTOXICITY	70
5.3 STATISTICAL ANALYSIS	71
DISCUSSION	71
6. SUMMARY AND CONCLUSIONS	74
REFERENCES	75

LIST OF ABBREVIATION

AO	Acridine orange
DCFH-DA	2', 7'-dichlorodihydrofluoresceindiacetate
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 β	Interleukin-1 beta
IL-6	Interleukin-6
PBS	Inferior vena cava
PI	Propidium iodide
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
TNF- α	Tumour necrosis factor alpha

LIST OF FIGURES

FIGURE NO	FIGURE NAME	PAGE NUMBER
1.1	Cancer cell	3
1.2	Cancer cell Characteristics	8
1.3	Cancer cell Development	9
1.4	Cancer and metabolism interact at many level	11
1.5	Cancer death cases	14
1.6	Incident Rates	15
1.7	Liver anatomy and physiology	18
1.8	Healthy liver and Cirrhotic liver	19
1.9	The role of immune cell and hepatocellular cancer	20
1.10	Stages of liver cancer	24

1.11	Symptoms of liver cancer	25
1.12	Treatment of liver cancer	27
1.13	Diagnosis and treatment of liver cancer	29
1.14	Structure of monacolin-x	31
4.1	HepG2 cell line	43
4.2	Schematic representation of monacolin-x	46
4.3	Cytotoxicity Assay	47
4.4	RNA Isolation	49
4.5	cDNA conversion	51
4.6	In vivo developmental toxic level of zebrafish using monacolin-x	55
4.7	Zebrafish life cycle	56

5.1	Dissolved Drug	58
5.2	96 well plate, after adding MTT	59
5.3	The MTT assay result of fibroblast cell line	59
5.4	The MTT assay result of HepG2 cell line	60
5.5	The Necrosis assay result of fibroblast cell line	61
5.6	The Necrosis assay results of HepG2 cell line	62
5.7	The Apoptosis assay result of fibroblast cell line	63
5.8	The Apoptosis assay result of HepG2 cell line	64
5.9	The DCFH-DA staining result of HepG2 cell line	65
5.10	The result of DPPH assay on HepG2 cell line	66
5.11	Before and After Treatment of liver cancer cell line	67

5.12	6 well plate used in the treatment of RNA isolation	67
5.13	RNA isolated	68
5.14	cDNA conversion	68
5.15	Apoptosis gene expression by qPCR analysis	69
5.16	Toxic effect of monacolin-x on the development of zebrafish embryos	70
5.17	Toxic effect of monacolin-x on the development of zebrafish embryos	71

LIST OF TABLES

TABLE NUMBER	TABLE NAME	PAGE NUMBER
4.1	List of cell line used	44
4.2	Inflammatory gene expression by qPCR analysis	51
4.3	Apoptosis gene expression by qPCR analysis	52

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a broad category of disorders that can originate in nearly any organ or tissue in the body when aberrant cells proliferate out of control, cross normal boundaries to infect other body parts, or spread to other organs. The latter process, known as metastasizing, is a primary contributor to cancer-related deaths. Other names for cancer that are commonly used are neoplasm and malignant tumour (*Brown et al., 2023*). Normally, cells develop and replicate to divide into new cells when the body needs them. As cells age or sustain damage, new ones take their place (*Khan and Farhana, 2024*). Sometimes this carefully regulated system breaks down, allowing damaged or abnormal cells to multiply and spread when they shouldn't. Geographical locations and cancer types have different incidence rates. The most common types of cancer in the globe are stomach, colorectal, prostate, lung, and breast cancer (*Bray et al., 2018*). On the other hand, depending on the population and geography, incidence rates for specific cancer types may vary significantly. Having certain infections (such the hepatitis and HPV viruses) and smoking are among the risk factors for cancer. Other risk factors include not exercising, eating poorly, consuming excessive alcohol, being fat, and being exposed to toxins (*Anand et al., 2008*). Regular screenings and early detection are crucial for improving outcomes and reducing the death rates associated with many malignancies. Screening tests including mammograms, colonoscopies, Pap smears, and others help detect cancer early and more effectively. With the advancement of cancer treatment, there are now more options than ever, such as targeted (*Altorki et al., 2023*). The human body, consisting of trillions of cells, can develop cancer virtually anyplace. As the body requires them, human cells normally divide and expand to generate new cells. Replicated cells replace old ones when they age or sustain damage (*Hammarlund et al., 2020*). Advances in genomics and molecular profiling have enabled the development of precision medicine approaches for cancer treatment. By tailoring therapy based on the genetic characteristics of individual tumour, precision

medicine aims to improve treatment outcomes and minimize side effects (Wang and Wang, 2023).

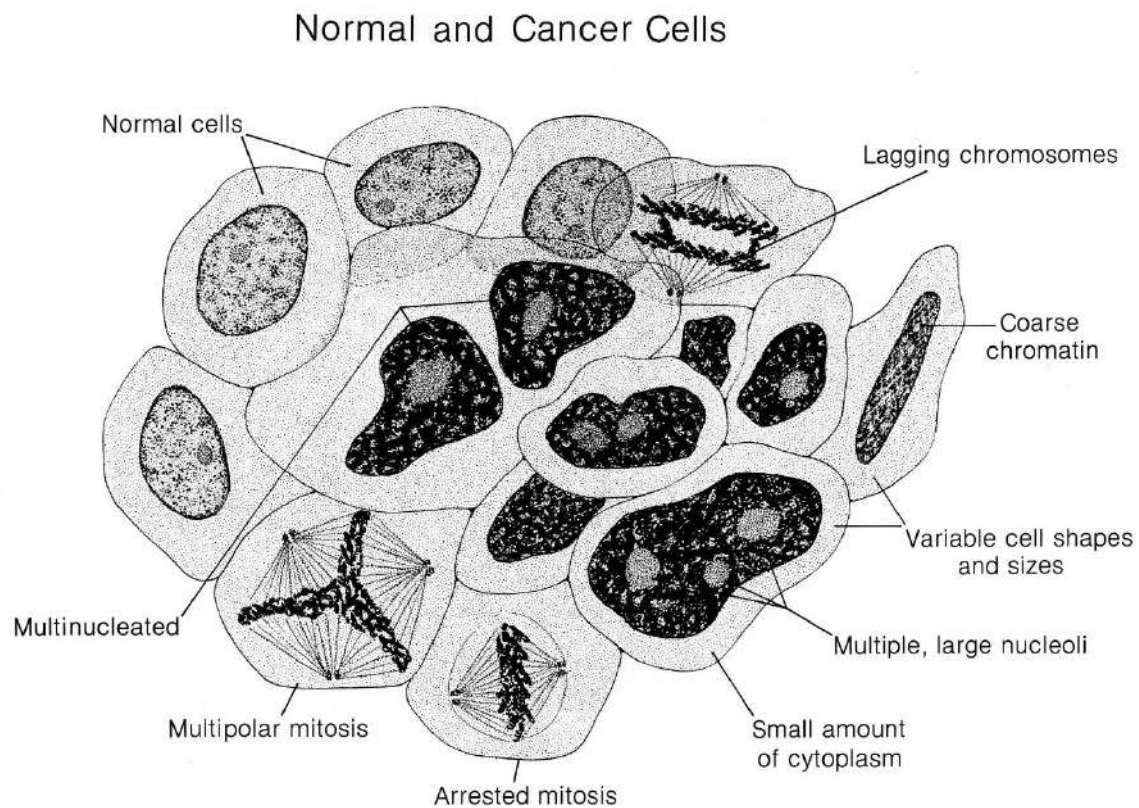


Fig:1.1 Cancer cell (Dominiak et al., 2020).

1.1.2 Cancer cell characteristics

There are many differences between cancer cells and normal cells in noncancerous (benign) or cancerous (malignant) tumours (Mirzaei et al., 2022). The major differences between normal cells and cancer cells relate to growth, communication, cell repair and death, "stickiness" and spread, appearance, maturation, evasion of the immune system, function and blood supply. When there are enough healthy cells to cover the hole in the skin, the production of new cells ceases and the incision is repaired by existing cells (Tang and Marshall, 2017). When there are sufficient cells, cancer cells, on the other hand, continue to develop. Before they have a chance to develop fully, cancer cells multiply quickly. Tumours frequently arise as a result of this persistence in replication (Mercadante and Kasi, 2024). Cell communication is required for

proper cellular activities or movements, and both failure and excess of this cross-talk can lead to tissue pathology. Normal and cancerous cells dynamically transmit reciprocal information, and, by contacting the tumour stromal cells, acquire a pro-tumoral phenotype that can promote cancer progression (*Dominiak et al., 2020*). Currently, many studies have documented that the vital role in tumour progression plays on a complex system of intercellular communication via direct cell-to-cell contact or through classical paracrine/endocrine signalling. The most common type of signal transition to neighbouring or long-distance cells is the secretion of soluble factors into the extracellular space, like cytokines, chemokines, and growth factors (*Torralba et al., 2016; Valenti et al., 2021*). Understanding the mechanism of cell–cell communication and cross-talk between a tumour and its microenvironment is of great importance for the development of effective cancer treatments (*Pasquier et al., 2013*). Normal tissues contain a multitude of cell types, molecular signals, and microenvironmental features that work in symphony to ensure tissue function, as well as maintain homeostasis. This homeostasis is dynamic and naturally emerges from the interplay between life and the environment (*Dyke and Weaver, 2013*). Homeostasis is the body's way of ensuring all its processes are working properly. Homeostasis is one area where normal cells and cancer cells are very different. To maintain normal cell homeostasis, normal cells are either repaired or die when they are damaged or get old. Cancer cells are either not repaired or do not undergo apoptosis (*Elmore, 2007*). One protein, p53, for instance, is responsible for determining if a cell is too damaged to be repaired and, if so, advising the cell to commit suicide. Damaged or aged cells might proliferate if the p53 protein is aberrant or inactive. One class of tumour suppressor gene that codes for proteins that inhibit cell growth is the p53 gene (*Ozaki and Nakagawara, 2011*). Regular cells release chemicals that cause them to adhere to one another as a clump. Since cancer cells are unable to produce these chemicals, they can float away to neighbouring places or travel through the circulation or lymphatic system to other parts of the body. Normal cells stay in the area of the body where they belong. For example, lung cells remain in the lungs. Some cancer cells may lack the adhesion molecules that cause stickiness, and are able to detach and travel via the bloodstream and lymphatic system to other regions of the body they have the ability to spread. Once they arrive in a new region (such as lymph nodes, the lungs, the liver, or the bones)

they begin to grow, often forming tumours far removed from the original tumour (Wang *et al.*, 2021; Xie *et al.*, 2022).

Cancerous and healthy cells can appear very different under a microscope. Cancer cells frequently show significantly greater variety in their sizes compared to normal cells; some of them are larger and some smaller than usual. Furthermore, both the cell and the nucleus, or brain of a cancerous cell, frequently have aberrant shapes. In comparison to regular cells, the nucleus appears larger and darker. Cancer cell nuclei contain more DNA than is necessary, which explains the blackness. When examined up close, cancer cells frequently have an unusually high number of chromosomes dispersed randomly (Kumar *et al.*, 2015). Regular cells develop. Cancer cells are immature because they proliferate quickly and divide before reaching full maturity. In contrast to differentiated, which refers to more mature cells, doctors refer to young cells as undifferentiated. An alternative interpretation of this would be to regard cancer cells as cells that never "grow up" and become adult-specific cells. The level of cell maturity is correlated with the cancer's grade. On a scale of 1 to 3, the most aggressive types of cancer are graded (Afify and Seno, 2019). The immune response is the intra organism defence mechanism that responds to threats to self. This defensive reaction takes place in three stages: recognition, processing, and reaction. Nonself elements are recognized as foreign substances by cells or active molecules involved in the immune response. Cells or active molecules involved in innate immunity recognize molecular patterns of nonself antigens (Kim and Cho, 2022). When normal cells become damaged, the immune system identifies and removes them. Cancer cells are able to evade the immune system long enough to grow into a tumour by either escaping detection or by secreting chemicals that inactivate immune cells that come to the scene (Vanneman and Dranoff, 2012). Normal cells perform the function they are meant to perform, whereas cancer cells may not be functional. For example, normal white blood cells help fight off infections. In leukemia, the number of white blood cells may be very high, but since the cancerous white blood cells are not functioning as they should, people can be more at risk for infection even with an elevated white blood cell count. The same can be true of substances produced. For example, normal thyroid cells produce thyroid hormones. Cancerous thyroid cells (thyroid cancer) may not produce thyroid

hormone. In this case, the body may lack enough thyroid hormone (hypothyroidism) despite an increased amount of thyroid tissue (*Carter et al., 2014*). Angiogenesis and lymph angiogenesis, which are initiated by chemical signals from tumour cells during a phase of rapid growth, are essential for tumour growth and spread (*Folkman, 1971*). As a result, angiogenesis plays a significant role in the development of cancer. Tumour angiogenesis and neovascularization are essentially four-step processes. Initially, there is a localized injury to the tissues' basement membrane. There is hypoxia and quick damage. Secondly, angiogenic factors-stimulated endothelial cells move. Third, endothelial cells multiply and become stable (*Nishida et al., 2006*).

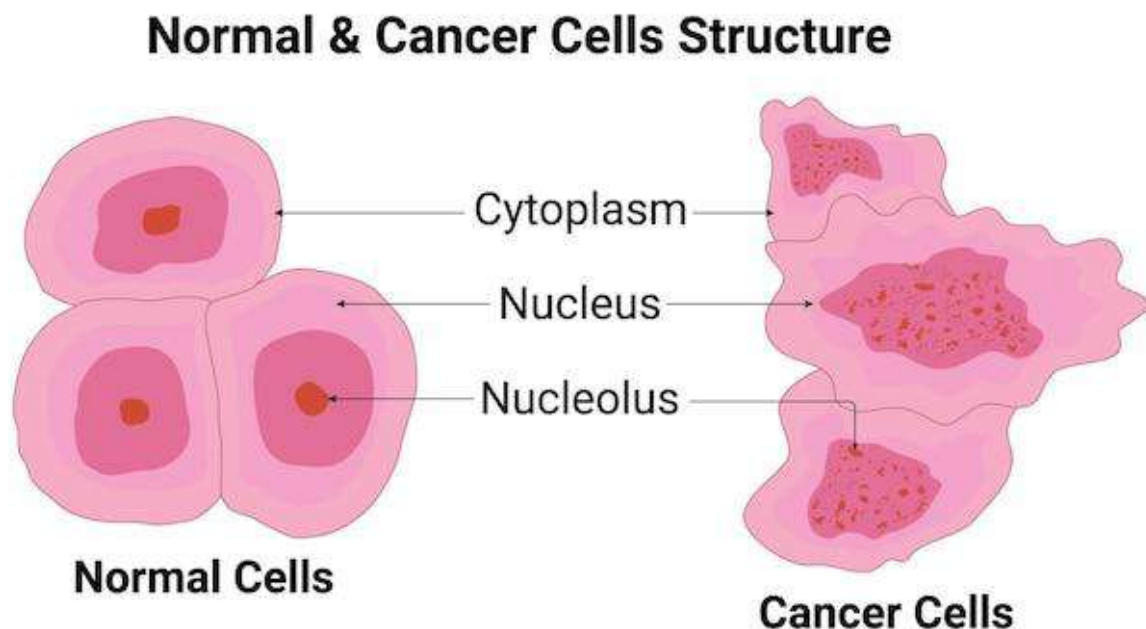


Fig:1.2 Cancer cell Characteristics (Vanneman and Dranoff, 2012).

1.1.3 Cancer Development

Cells become cancerous through a series of genetic mutations that disrupt the normal regulation of cell growth and division. Initiation is the initial step where a normal cell undergoes a mutation in its DNA. Mutations can be caused by various factors such as exposure to carcinogens genetic predisposition, or random errors

during DNA replication. These mutations can activate oncogenes or deactivate tumour suppressor genes (*Basu, 2018*). After the initiation step, the mutated cell may begin to divide more rapidly than normal cells, forming a small cluster or tumour. This stage involves further mutations that enable the abnormal cell to grow and survive more effectively. These mutations may involve alterations in signalling pathways that control cell growth and survival, allowing the cancerous cells to evade normal regulatory mechanisms (*Feitelson et al., 2015*). The cancer cells acquire additional mutations that enhance their ability to invade nearby tissues and spread to distant parts of the body (metastasis). These mutations may enable the cancer cells to penetrate blood vessels or lymphatic vessels, allowing them to travel to other organs and form secondary tumours (*Alexandrov et al., 2016*). Metastasis is a critical step in cancer development that involves the spread of cancer cells from the primary tumour to distant sites in the body. This process is facilitated by various mechanisms, including the ability of cancer cells to invade blood vessels or lymphatic vessels, survive in the circulation, and establish secondary tumours in distant organs or tissues. Metastasis is a major contributor to cancer-related morbidity and mortality (*Sarkar et al., 2013*).

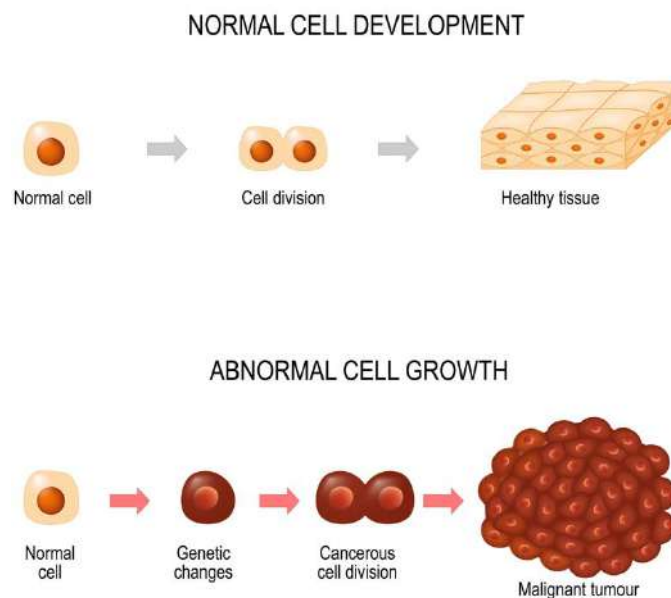


Fig:1.3 Cancer cell development (*Feitelson et al., 2015*)

1.1 4 Cancer cell metabolism

Cancer cells often exhibit a heightened uptake of glucose, which provides them with a constant source of energy and carbon for biomass synthesis (*Liberti and Locasale, 2016*). This phenomenon is exploited in diagnostic imaging techniques like positron emission tomography (PET), where radioactively labelled glucose analogues are used to detect tumours based on their increased glucose uptake. Cancer cells frequently rely on glycolysis, the breakdown of glucose into pyruvate, even in the presence of oxygen, rather than oxidative phosphorylation (OXPHOS) in mitochondria. This metabolic preference, known as the Warburg effect, allows cancer cells to rapidly produce ATP and generate metabolic intermediates needed for cell growth and proliferation. While cancer cells may exhibit reduced reliance on oxidative phosphorylation, they still utilize mitochondria for various metabolic processes. However, the function of mitochondria in cancer cells can be altered, with changes in mitochondrial dynamics, substrate utilization, and reactive oxygen species (ROS) production. Glutamine, an amino acid, serves as a major substrate for cancer cells to support biosynthetic pathways and maintain redox balance. Cancer cells often display increased glutamine uptake and utilization, converting glutamine into metabolites that contribute to nucleotide, lipid, and protein synthesis. Cancer cells frequently exhibit alterations in lipid metabolism to support their rapid proliferation and membrane synthesis. This may involve increased fatty acid synthesis, uptake of exogenous lipids, and alterations in lipid storage and utilization pathways. Cancer cells may become dependent on certain nutrients or metabolic pathways due to genetic mutations, microenvironmental cues, or therapeutic pressures. Targeting these dependencies has emerged as a promising strategy for cancer therapy, known as metabolic targeting or metabolic therapy. In order to produce nucleotides and NADPH, cancer cells often upregulate the PPP, a metabolic route that runs parallel to glycolysis. Lipid synthesis and antioxidant defence are two biosynthetic processes that depend on NADPH for redox balance (*Stincone et al., 2015*). Cancer cells frequently display mitochondrial malfunction and metabolic reprogramming, even though they still depend on them for several metabolic functions. This may entail modifications to the structure, metabolism, and function of the mitochondria, increasing the reliance on glycolysis to produce ATP. Oxidative stress is heightened in cancer cells as a result of increased

metabolic activity and malfunctioning mitochondria. Their ability to preserve redox equilibrium and encourage cell viability depends on antioxidant mechanisms. The metabolism of cancer can fluctuate significantly between tumours types and even within the same tumour. When faced with stimuli from the surrounding environment, nutritional availability, and therapeutic interventions, tumours cells may modify their metabolic pathways (Chaiswing *et al.*, 2018; Wondrak, 2009).

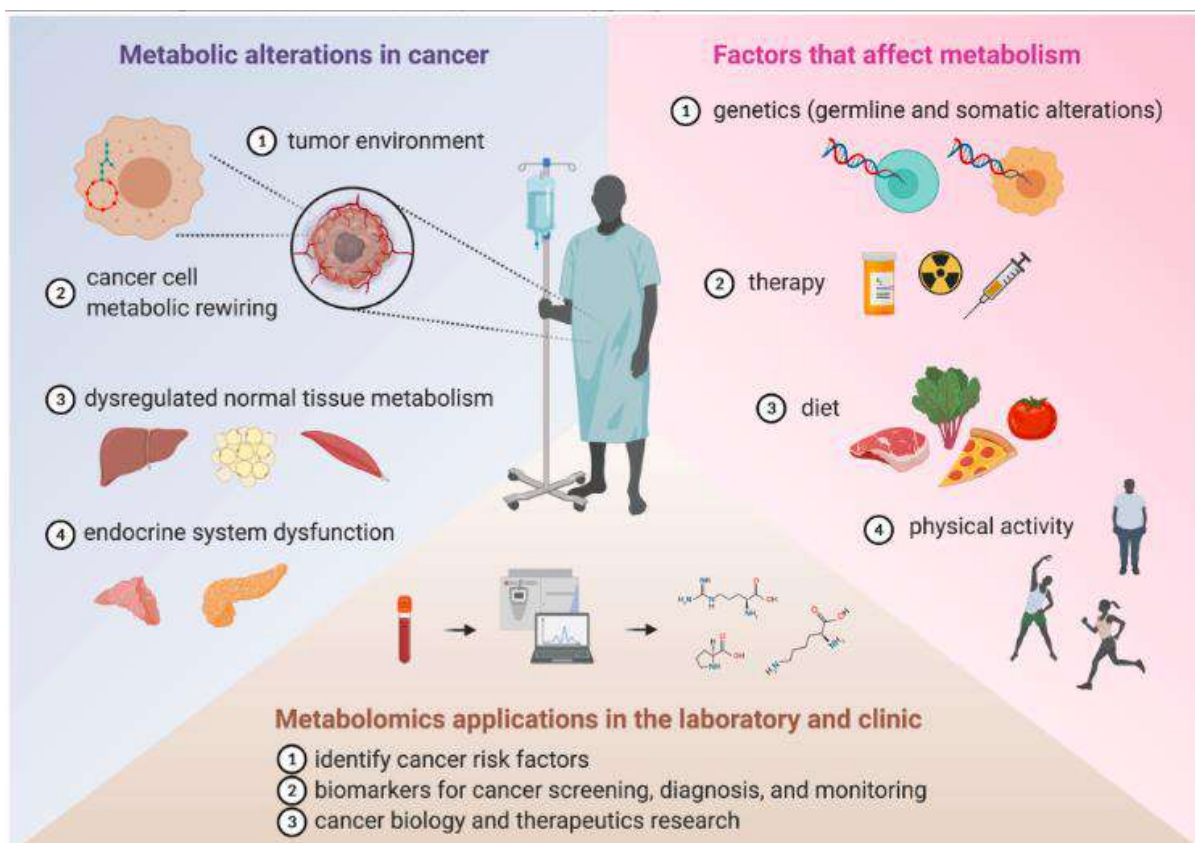


Fig:1.4 Cancer and metabolism interact at many levels. Cancer causes metabolic alterations in cancer cells and normal tissues, which, in turn, interact with intrinsic and extrinsic factors to affect systemic metabolism. Metabolomics is a systems-based approach used to define these complex metabolic interactions for diagnostic and therapeutic gain (Schmidt *et al.*, 2021).

1.1.5 Function of Cancer

Cancer cells, like normal cells, are part of the body's complex biological system. However, they exhibit several key characteristics that differentiate them from

normal cells. Cancer cells have lost the ability to regulate their growth and division. They continuously divide and proliferate, forming tumors or masses of abnormal cells (*Mercadante and Kasi, 2024*). Through a process known as metastasis, cancer cells can invade neighboring tissues and move to distant parts of the body (*van Zijl et al., 2011*). This ability to spread to other organs is a hallmark of cancer and makes it more difficult to treat. Cancer cells can evade programmed cell death, which is a mechanism that normally eliminates damaged or abnormal cells (*Gutschner and Diederichs, 2012*). This resistance to cell death allows cancer cells to survive and proliferate unchecked (*Pfeffer and Singh, 2018*). Cancer cells can induce the formation of new blood vessels to supply nutrients and oxygen to tumors. This process helps tumors grow and spread (*Saman et al., 2020*). Cancer cells often have abnormalities in their DNA, leading to genomic instability (*Ferguson et al., 2015*). This instability can result in further genetic mutations, promoting tumor progression and heterogeneity. Cancer cells can evade detection and destruction by the immune system through various mechanisms, such as downregulating immune responses or expressing molecules that inhibit immune cell function (*Kim and Cho, 2022*). Cancer cells undergo metabolic changes to support their rapid growth and proliferation. They often rely on glycolysis (a process that breaks down glucose) even in the presence of oxygen, known as the Warburg effect. Cancer cells interact with their surrounding microenvironment, including nearby cells, extracellular matrix, and signaling molecules, to promote tumor growth, invasion, and metastasis (*Liberti and Locasale, 2016*).

1.1.6 Incidence

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020. The estimated number of incident cases of cancer in India for the year 2022 was found to be 14,61,427 (crude rate:100.4 per 100,000). In India, one in nine people are likely to develop cancer in his/her lifetime (*Sathishkumar et al., 2022*). The number of people living with cancer (either recently diagnosed or surviving) is also on the rise due to advancements in early detection and treatment (*Pulumati et al., 2023*). Each year, the American Cancer Society estimates the numbers of new cancer cases and deaths in the United States and compiles the most recent data on population-based cancer occurrence and outcomes using

incidence data collected by central cancer registries and mortality data collected by the National Centre for Health Statistics. In 2023, 1,958,310 new cancer cases and 609,820 cancer deaths are projected to occur in the United States. Cancer incidence increased for prostate cancer by 3% annually from 2014 through 2019 after two decades of decline, translating to an additional 99,000 new cases; otherwise, however, incidence trends were more favourable in men compared to women. For example, lung cancer in women decreased at one half the pace of men (1.1% vs. 2.6% annually) from 2015 through 2019, and breast and uterine corpus cancers continued to increase, as did liver cancer and melanoma, both of which stabilized in men aged 50 years and older and declined in younger men. However, a 65% drop in cervical cancer incidence during 2012 through 2019 among women in their early 20s, the first cohort to receive the human papillomavirus vaccine, foreshadows steep reductions in the burden of human papillomavirus-associated cancers, the majority of which occur in women. Despite the pandemic, and in contrast with other leading causes of death, the cancer death rate continued to decline from 2019 to 2020 (by 1.5%), contributing to a 33% overall reduction since 1991 and an estimated 3.8 million deaths averted. This progress increasingly reflects advances in treatment, which are particularly evident in the rapid declines in mortality (approximately 2% annually during 2016 through 2020) for leukemia, melanoma, and kidney cancer, despite stable/increasing incidence, and accelerated declines for lung cancer (*Sathishkumar et al., 2022*).

Cancer is one of the leading causes of morbidity and mortality globally. According to the World Health Organization (WHO), there were an estimated 19.3 million new cancer cases and 10 million cancer-related deaths worldwide in 2020. These numbers continue to rise, driven by factors such as population growth, aging, lifestyle changes, and improvements in cancer detection and reporting. The most common types of cancer worldwide vary by region but generally include lung cancer, breast cancer, colorectal cancer, prostate cancer, and stomach cancer. However, the incidence rates of specific cancers can vary significantly between countries and regions due to differences in risk factors, environmental exposures, genetic predisposition, and access to healthcare. Cancer is among the leading causes of death worldwide. In 2018, there were 18.1 million new cases and

9.5 million cancer-related deaths worldwide. By 2040, the number of new cancer cases per year is expected to rise to 29.5 million and the number of cancer-related deaths to 16.4 million. Generally, cancer rates are highest in countries whose populations have the highest life expectancy, education level, and standard of living. But for some cancer types, such as cervical cancer, the reverse is true, and the incidence rate is highest in countries in which the population ranks low on these measures.

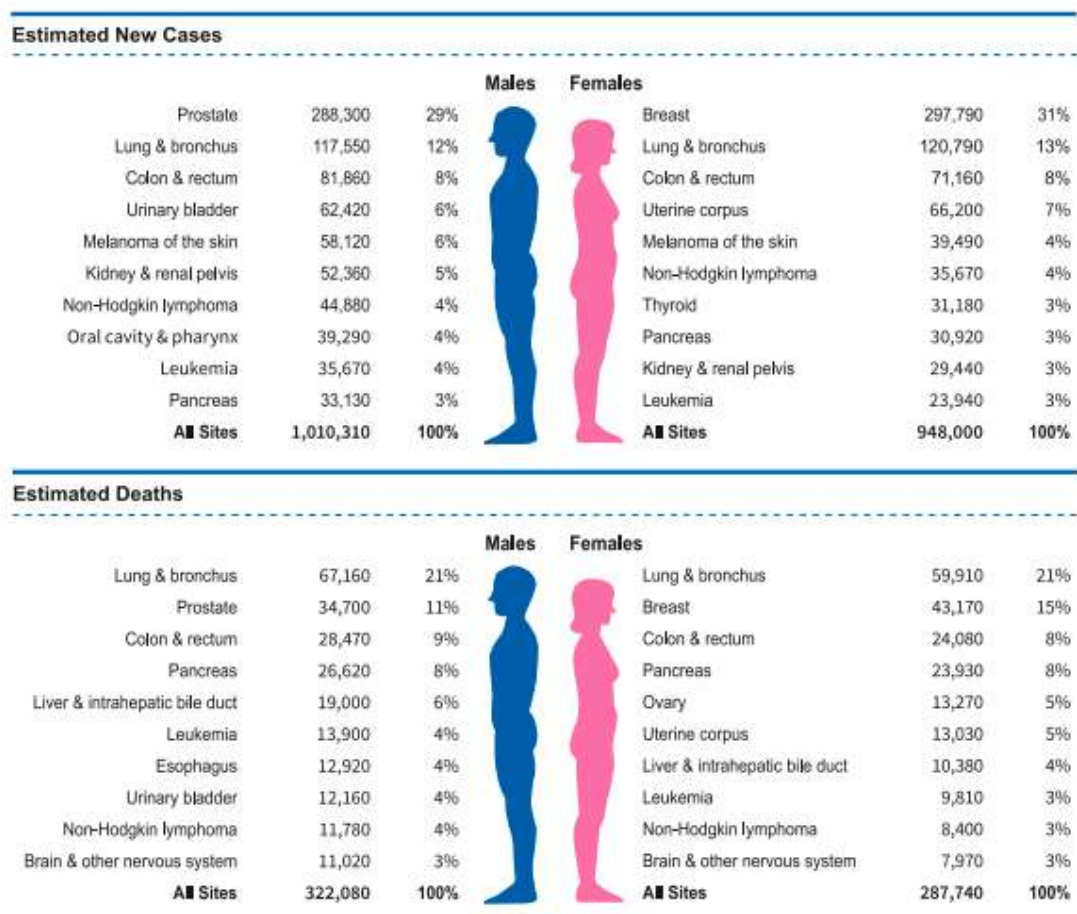


Fig:1.5 Ten leading cancer types for the estimated new cancer cases and deaths by sex, United States, 2023. Estimates are rounded to the nearest 10, and case sex include basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder (Siegel et al., 2023).

Globally, there are significant regional differences in the incidence and prevalence of cancer. There are some places with greater overall cancer rates than others, including Western Europe, North America, and some portions of Asia. Additionally, local risk factors and environmental exposures may contribute to a higher incidence of a particular kind of cancer in a certain area. The worldwide cancer burden is influenced by a number of common risk factors, such as alcohol consumption, unhealthy diet and obesity, physical inactivity, exposure to environmental carcinogens (e.g., air pollution, occupational hazards), infections (e.g., helicobacter pylori, HPV, hepatitis B and C viruses), and genetic predisposition. Implementing comprehensive cancer control programs and promoting cancer prevention techniques (like tobacco use) are some of the steps being taken to lessen the worldwide burden of cancer (*Garner et al., 2023*).

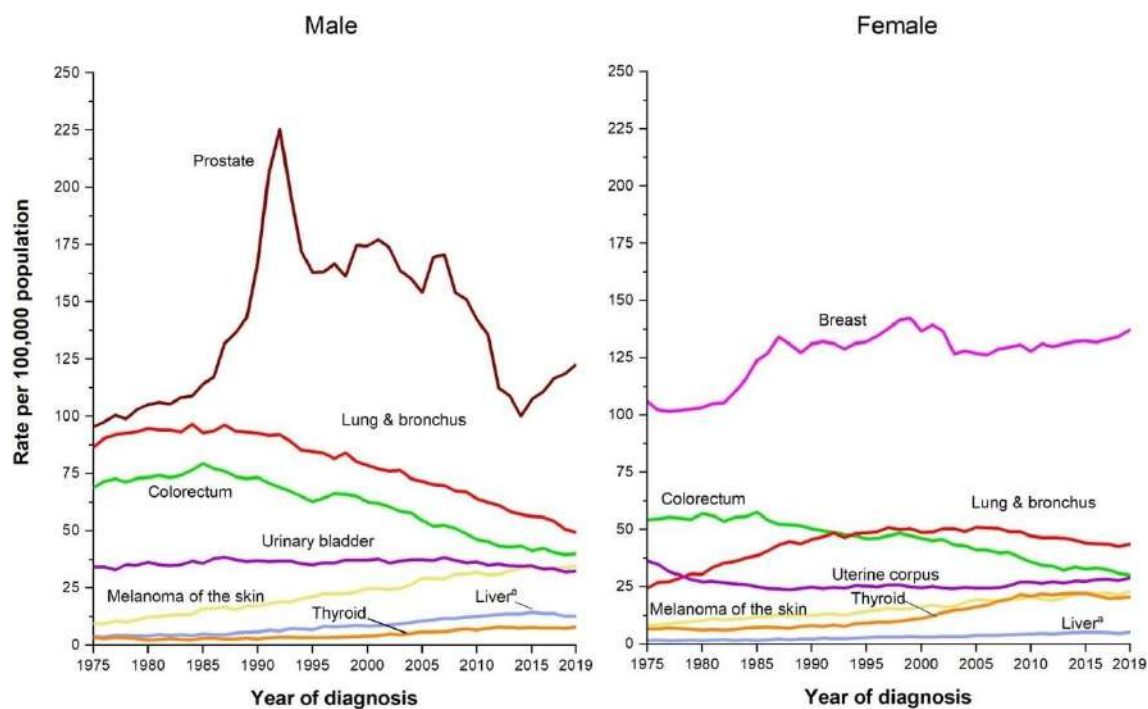


Fig:1.6 Trend sin incidence rates for selected cancers by sex, United States, 1975-2019.Rates are age adjusted to the 2000 US standard population and adjusted for delays in reporting. Liver includes in therapeutic bile duct (*Cui et al., 2024*).

1.1.7 Treatment of Cancer

The treatment of cancer varies widely depending on factors such as the type and stage of cancer, as well as the patient's overall health and preferences (*Debela et al., 2021*). Common treatment modalities include as Surgery involves the physical removal of cancerous tumours and surrounding tissues. It is often used when the cancer is localized and has not spread to other parts of the body. Chemotherapy uses drugs to kill cancer cells or stop them from growing. It can be administered orally or intravenously and is often used to treat cancers that have spread or are difficult to remove with surgery. Radiation therapy uses high-energy rays or particles to destroy cancer cells (*Baskar et al., 2014*). It can be used as a standalone treatment or in combination with surgery or chemotherapy (*Debela et al., 2021*). Immunotherapy works by harnessing the body's immune system to fight cancer. It includes treatments such as checkpoint inhibitors, CAR-T cell therapy, and monoclonal antibodies (*Kumar et al., 2021*). Targeted therapy targets specific molecules or pathways involved in the growth and spread of cancer cells. It is often used in cancers that have specific genetic mutations or molecular abnormalities. Hormone therapy is used to treat cancers that are sensitive to hormones, such as breast and prostate cancers. It works by blocking the body's production of certain hormones or by interfering with the hormones' effects on cancer cells. Stem cell transplant, also known as bone marrow transplant, may be used in certain cases to replace diseased or damaged bone marrow with healthy stem cells. It is often used in the treatment of blood cancers like leukemia and lymphoma (*Khaddour et al., 2024*). Palliative care focuses on relieving symptoms and improving the quality of life for patients with advanced or terminal cancer. It can be provided alongside curative treatments or as the main focus of care for patients whose cancer cannot be cured. Treatment plans are often individualized based on the specific characteristics of the cancer and the patient's preferences (*Krzyszczuk et al., 2018; Yeo et al., 2023*).

1.1.8 Chemotherapy

Chemotherapy is a common treatment for cancer that involves the use of drugs to kill cancer cells or stop them from growing and spreading (*Amjad et al., 2024*). Chemotherapy drugs can be administered in various ways, including orally, intravenously (IV), intramuscularly (IM), or topically, depending on the type of

cancer and the treatment plan. There are many different types of chemotherapy drugs, each with its own mechanism of action and side effects (*Anand et al., 2023*). These drugs may be used alone or in combination to treat cancer. Chemotherapy may be given in cycles, with periods of treatment followed by rest periods to allow the body to recover. The frequency and duration of chemotherapy cycles vary depending on the type and stage of cancer, as well as the individual patient's response to treatment. Adjuvant chemotherapy is given after surgery or other primary treatments to kill any remaining cancer cells and reduce the risk of cancer recurrence (*Beccari et al., 1986*). Neoadjuvant chemotherapy is given before surgery or radiation therapy to shrink tumours and make them easier to remove. Often, chemotherapy drugs are used in combination with other cancer treatments such as surgery, radiation therapy, immunotherapy, or targeted therapy to increase effectiveness (*Korde et al., 2021*). Chemotherapy can cause a range of side effects, including nausea and vomiting, hair loss, fatigue, increased risk of infection, and changes in blood cell counts. However, not all patients experience the same side effects, and many can be managed or minimized with supportive care. During chemotherapy treatment, patients are regularly monitored by their healthcare team to assess treatment response and manage side effects (*Rao and Faso, 2012*). Supportive care measures such as anti-nausea medications, pain management, nutritional support, and psychological support are often provided to help patients cope with the physical and emotional challenges of treatment. Some chemotherapy drugs can cause long-term side effects or complications, such as damage to the heart, lungs, nerves, or reproductive organs. Close monitoring and follow-up care are essential for managing these potential risks (*Pinho et al., 2002*).

1.2 Anatomy and Physiology of the Human Liver

The human liver is a vital organ located in the upper right portion of the abdomen, beneath the diaphragm and above the stomach. It is the largest internal organ and plays a crucial role in many bodily functions. The anatomy of the liver is a reddish-brown organ with a rubbery texture. It is divided into two main lobes: the right lobe and the smaller left lobe. These lobes are further divided into smaller lobules (*Zaefarian et al., 2019*). The function of the liver is the liver regulates the metabolism of nutrients, including carbohydrates, proteins, and fats. It processes nutrients absorbed from the digestive system, stores energy in the form of

glycogen, and converts excess glucose into glycogen for storage (Rui, 2014). The liver filters and detoxifies harmful substances such as drugs, alcohol, and metabolic waste products, converting them into less harmful forms for excretion. The liver produces bile, a digestive fluid that helps emulsify fats and aid in their digestion and absorption in the small intestine. The liver stores vitamins (such as A, D, E, K, and B12), minerals (such as iron and copper), and glycogen for later use by the body. The liver synthesizes proteins, including blood-clotting factors, albumin, and various hormones. The liver receives a dual blood supply from the hepatic artery and the portal vein. Oxygen-rich blood from the hepatic artery provides oxygen to the liver cells, while nutrient-rich blood from the portal vein carries digested nutrients from the intestines to the liver for processing (Kalra et al., 2024). The liver has remarkable regenerative abilities. It can regenerate lost or damaged tissue, allowing it to recover from injury or surgical removal of a portion of the liver. The liver can be affected by various diseases and conditions, including hepatitis, cirrhosis, fatty liver disease, liver cancer, and liver failure. These conditions can impair liver function and may require medical treatment or interventions such as lifestyle changes, medications, or surgery (Hora and Wuestefeld, 2023).

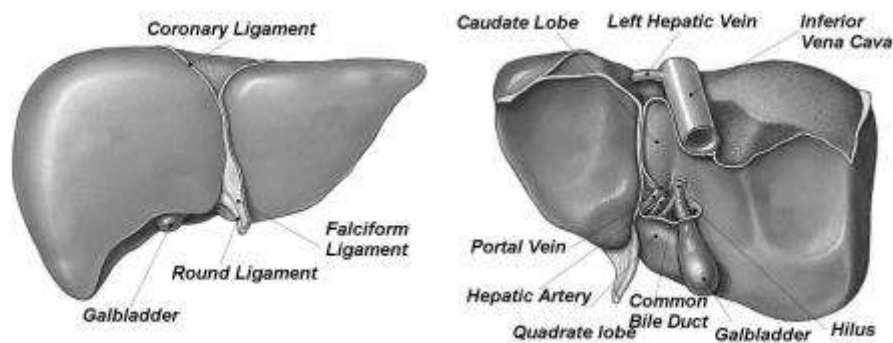


Fig:1.7 Liver Anatomy and Physiology (Kalra et al., 2024)

1.3 Liver Cancer

Liver cancer, also known as hepatic cancer, refers to the abnormal growth of cells in the liver (Chidambaranathan-Reghupaty et al., 2021). There are different types of liver cancer, but the most common form is hepatocellular carcinoma (HCC), which begins in the main type of liver cells called hepatocytes. Other types of liver cancer include intrahepatic cholangiocarcinoma and hepatoblastoma, which are

less common (*Jiang et al., 2018*). Liver cancer is cancer that begins in the cells of your liver. Your liver is a football-sized organ that sits in the upper right portion of your abdomen, beneath your diaphragm and above your stomach. Several types of cancer can form in the liver. The most common type of liver cancer is hepatocellular carcinoma, which begins in the main type of liver cell (hepatocyte). Cancer that spreads to the liver is more common than cancer that begins in the liver cells (*Griscom and Wolf, 2024*). Cancer that begins in another area of the body such as the colon, lung or breast and then spreads to the liver is called metastatic cancer rather than liver cancer. This type of cancer is named after the organ in which it began such as metastatic colon cancer to describe cancer that begins in the colon and spreads to the liver (*Seyfried and Huysentruyt, 2013*). Liver cancer is the 6th most common cancer worldwide. It is the 5th most common cancer in men and the 9th most common cancer in women (*Mun et al., 2023*).

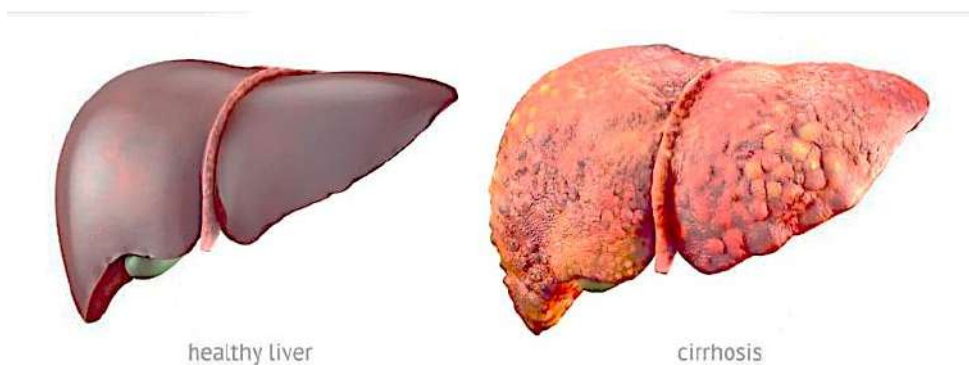


Fig:1.8 Healthy and cirrhotic human liver (*Mattiuzzi and Lippi, 2019*)

1.3.1 Liver Metabolism

Liver cancer cells exhibit increased glucose uptake and glycolysis, a phenomenon known as the Warburg effect. Even in the presence of oxygen, cancer cells preferentially utilize glycolysis for energy production, leading to increased lactate production (*Jang et al., 2013*). This metabolic shift is facilitated by the upregulation of glycolytic enzymes such as hexokinase, phosphofructokinase, and lactate dehydrogenase. Glutamine is a non-essential amino acid that serves as a major substrate for energy production and biosynthesis in cancer cells (*Schiliro and Firestein, 2021*). Liver cancer cells often display enhanced glutamine uptake and utilization to support cell proliferation and survival. Glutamine can be converted to

glutamate and subsequently into α -ketoglutarate, an intermediate of the tricarboxylic acid (TCA) cycle, to fuel ATP production and biosynthetic pathways (Altman et al., 2016). Dysregulated lipid metabolism is a common feature of liver cancer. Cancer cells exhibit increased lipogenesis to meet the demands for membrane synthesis and energy production (Lukey et al., 2013). This involves the upregulation of enzymes such as fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC). Additionally, liver cancer cells can enhance fatty acid uptake from the microenvironment to support their growth (Vanauberg et al., 2023). Despite the reliance on glycolysis, liver cancer cells can also maintain functional mitochondria and oxidative phosphorylation (Pan et al., 2021). However, the contribution of OXPHOS to ATP production may be reduced compared to normal hepatocytes. Mitochondrial dysfunction and alterations in mitochondrial metabolism have been reported in liver cancer, contributing to tumour progression and therapy resistance. Liver cancer cells often exhibit alterations in one-carbon metabolism, which plays a crucial role in nucleotide biosynthesis, methylation reactions, and redox balance. Aberrant expression of enzymes involved in one-carbon metabolism, such as serine hydroxy methyltransferase (SHMT) and methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), has been observed in liver cancer and is associated with tumour growth and metastasis (Shuvalov et al., 2017).

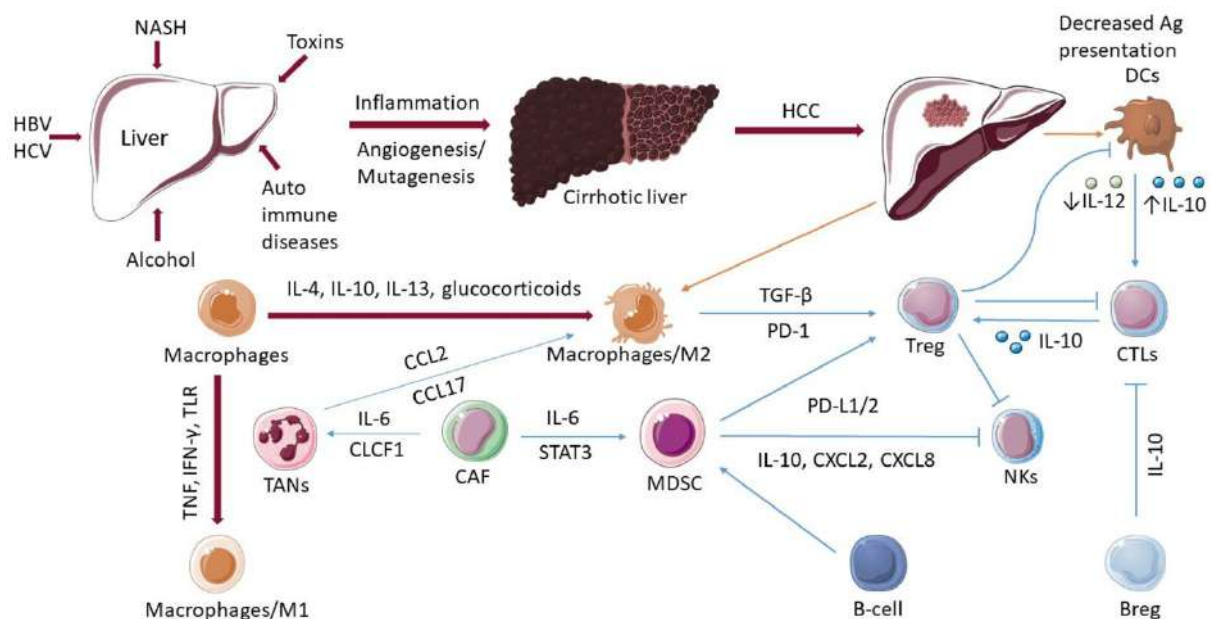


Fig:1.9 The role of immune cells in hepatocellular cancer. In the development of HCC, various factors play a decisive role such as hepatitis B

virus (HBV), hepatitis C virus (HCV), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), toxins, and autoimmune diseases. The actions of numerous immune cells become dysregulated as the disease develops from cirrhosis of the liver to hepatocellular carcinoma (HCC). The ability of dendritic cells (DCs) to present antigens is decreased. The activation of CD8⁺ T-cells (CTLs) is dependent on the activation of a DCs. Macrophages differentiate into an “alternatively-activated phenotype”, M2, which promotes the recruitment and growth of regulatory T-cells (Tregs). Tregs have a negative impact on CTLs, DCs, and natural killer (NK) cells. M1 macrophages are activated by microbial components or proinflammatory cytokines (TNF, IFN- γ , TLR). Tumour-associated neutrophils (TANs) produce the chemokines, and CCL2 and CCL17 were observed to recruit tumour-associated macrophages (TAMs). Cancer-associated fibroblasts (CAFs) induce activation of TANs via IL-6 and CLCF1 and promote the differentiation of monocytes into MDSCs via IL-6-mediated STAT3 activation. B cells can be associated with pro-tumorigenic processes by activating MDSCs. Regulatory B-cells promote T-cell dysregulation through an IL10-dependent mechanism (Suresh et al., 2020).

1.3.2 Statistics

Liver cancer is the fifth most common cause of cancer death in Men and the seventh most common cause of cancer death among women. Although the overall liver cancer death rate more than doubled since 1980, it stabilized between 2016 and 2020 for both men and women. In 2023, an estimated 41,210 adults (27,980 men and 13,230 women) in the United States will be diagnosed with primary liver cancer. Since 1980, the incidence of liver cancer has more than tripled. However, between 2014 and 2015, incidence rates slowed in women while remaining steady in men. Men are almost 3 times more likely than women to be diagnosed with the disease. Worldwide, an estimated 905,677 people were diagnosed with liver cancer in 2020. It is estimated that 29,380 deaths (19,000 men and 10,380 women) from this disease will occur in the United States in 2023.

Latest liver cancer data

Liver cancer is the 6th most common cancer worldwide. It is the 5th most common cancer in men and the 9th most common cancer in women. There were more than 900,000 new cases of liver cancer in 2020. The 10 countries with the highest rates of liver cancer and the highest number of deaths from liver cancer in 2020 are shown in the tables below. ASR = age-standardised rates. These are a summary measure of the rate of disease that a population would have if it had a standard age structure. Standardisation is necessary when comparing populations that differ with respect to age because age has a powerful influence on the risk of dying from cancer.

Liver cancer rates

The following 3 tables show total global cancer incidence and rates in 2020, followed by the figures for men and women. Mongolia had the highest overall rate of liver cancer in 2020, followed by Egypt.

1.3.3 Stages of Liver Cancer

The stage of a cancer describes how much cancer is in the body. It helps determine how serious the cancer is and how best to treat it (*Ni and Zhang, 2020*).

Stage 1: At the onset of liver cancer, a tumour develops within the liver. However, effects of the tumour are localized, as it is yet to come in contact with any blood vessels (*Pinter et al., 2016*).

Stage 2: The tumour grows in dimension reaching a size up to 5 cm across. The possibility of developing several small tumours is also there. Though the tumour has grown into blood vessels, the spread is still localised (*Hillen and Griffioen, 2007*).

Stage 3: This stage is also characterised by outgrowth of the tumour(s). The average size of the tumour is 5 cm across, and surrounding lymph nodes still remain immune to the cancer (*De Marchi et al., 2020*).

Stage 3B: One or more tumours intrude into the portal or hepatic vein of the liver. Here the nearby lymph nodes and distant body organs are yet to get in contact with the malignancy (*Jiang et al., 2017*).

Stage 3C: The tumour has either engulfed the outer surface of the liver or penetrated into nearby organs. Distant sites are still not affected by the cancer.

Stage 4A: The size and number of tumours in the liver grow phenomenally. This stage also marks the spread of tumours into lymph nodes, blood vessels or surrounding organs. The distant sites are still unaffected (Leong *et al.*, 2022).

Stage 4B: The entire body is infected by the malignant cells (Yamakado and Hirota, 2015).

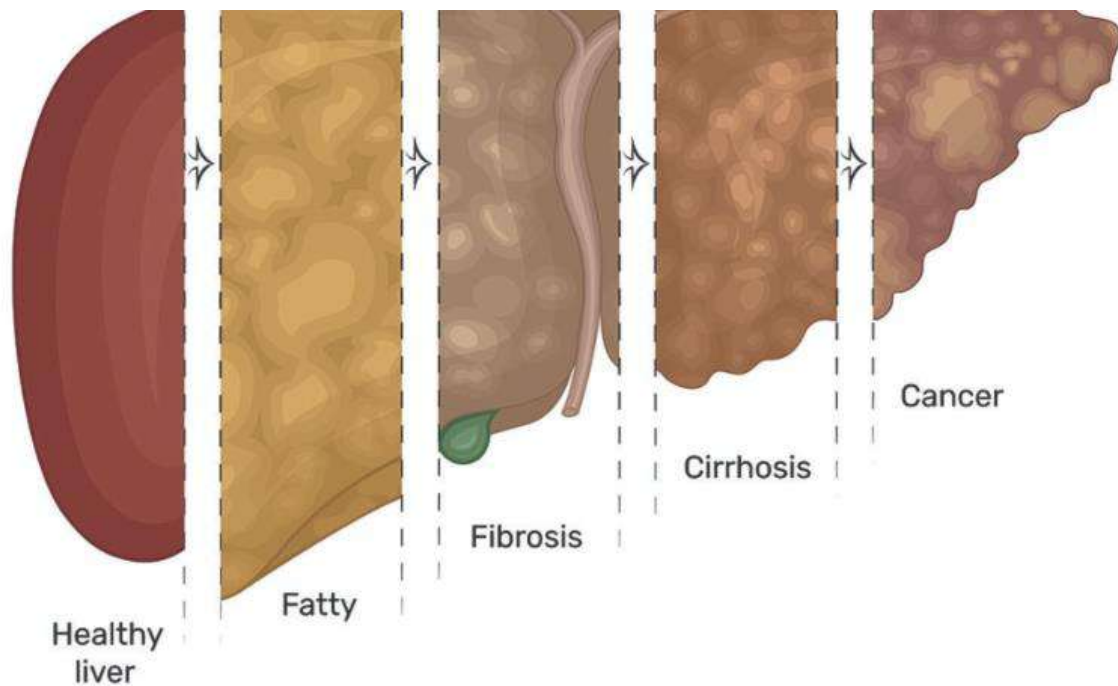


Fig:1.10 Liver cancer is divided into four stages based on the size of the tumour (Ni and Zhang, 2020).

1.3.4 Symptoms of Liver Cancer

Liver cancer symptoms mostly appear as the cancer increases or enters in its advanced stage. The following symptoms of shown in (Fig:1.11).

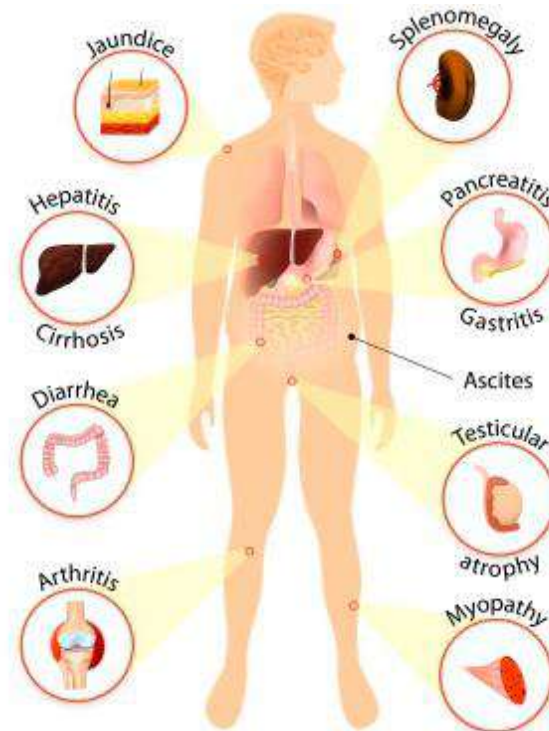


Fig:1.11 Symptoms of liver cancer (Chidambaranathan-Reghupaty et al., 2021)

1.3.5 Risk Factors

DNA mutations disturb the natural process of cell division giving rise to abnormal multiplication of the cells and eventually forms a malignant *tumour* (Torgovnick and Schumacher, 2015). Chronic viral infections including Hepatitis B virus (HBV) or hepatitis C virus (HCV) are the main cause of liver cancer. Alcohol-induced liver cirrhosis. Aflatoxin exposure can lead to the development of Hepatocellular carcinoma, HCC (Russo et al., 2022). Non-alcoholic fatty liver disease or genetic disorders such as haemochromatosis, or alpha 1-antitrypsin deficiency, Type 2 diabetes, Obesity, Tobacco smoking, Exposure to certain chemicals, High alcohol consumption (Benedict and Zhang, 2017).

1.4 Treatment of Liver Cancer

Treatment for liver cancer depends on several factors including the stage of the cancer, the patient's overall health, and the location and size of the tumour. Surgical options may include removing the tumour (partial hepatectomy) or a liver transplant in cases where the cancer is confined to the liver and the patient is eligible for transplantation and the Techniques like radiofrequency ablation (RFA),

microwave ablation, or ethanol injection can be used to destroy small liver tumours by applying extreme heat or cold directly to the cancer cells, and the radiation therapy is a best treatment for the cancer and this treatment uses high-energy rays to kill cancer cells (*Lurje et al., 2019*). Techniques like external beam radiation therapy or stereotactic body radiation therapy (SBRT) may be employed for liver cancer and another treatment of the cancer is transarterial chemoembolization and this procedure involves injecting chemotherapy drugs directly into the blood vessels supplying the tumour, followed by blocking those vessels to cut off the tumour's blood supply and Certain drugs target specific abnormalities present in cancer cells (*Lewis et al., 2022*). Drugs like sorafenib or lenvatinib may be used for advanced liver cancer. Drugs that stimulate the body's immune system to recognize and attack cancer cells are being studied and used in the treatment of liver cancer. While not always highly effective for liver cancer, chemotherapy may be used in some cases, especially when the cancer has spread beyond the liver. This involves treatments to manage symptoms and side effects of cancer, like pain management, nutritional support, and palliative care. Liver transplantation has proven to be the most effective treatment for patients with hepatocellular carcinoma, a common type of liver cancer. If a patient has liver disease, such as cirrhosis, liver transplantation can also further reduce further the risk of recurrence following treatment. Special needles are inserted directly through the skin or through an incision in the abdomen to reach the tumour. High-energy radio waves heat the needles and tumour which kills cancer cells. The tumour is exposed to high temperatures created by microwaves. This can damage and kill cancer cells or make them more sensitive to the effects of radiation and certain anticancer drugs. A small needle is used to inject ethanol (pure alcohol) directly into a tumour to kill cancer cells. Several treatments may be needed (*Garg et al., 2022*). Usually local anesthesia is used, but if the patient has many tumours in the liver, general anesthesia may be used. An instrument is used to freeze and destroy cancer cells. This type of treatment is also called cryotherapy and cryosurgery.

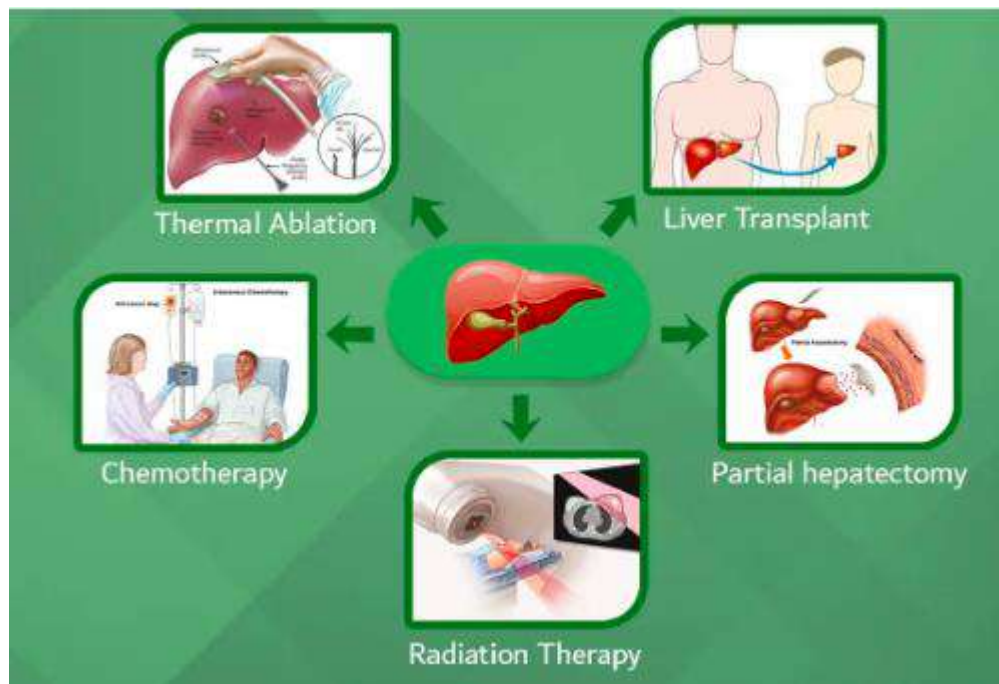


Fig:1.12 Treatment options for people with liver cancer are surgery (including a liver transplant), ablation, embolization, targeted therapy, radiation therapy, and chemotherapy. You may have a combination of treatments (Gosalia et al., 2017)

1.5 Chemotherapy

Chemotherapy is a powerful treatment approach that uses drugs to destroy cancer cells or slow down their growth. It's one of the most common treatments for cancer and can be used in different ways depending on the type and stage of cancer, as well as the patient's health condition (Anand et al., 2023). Chemotherapy drugs can be administered orally in the form of pills or intravenously through injections or infusion into a vein. These drugs travel throughout the body via the bloodstream, reaching cancer cells in different parts of the body. Chemotherapy can be used as the primary treatment for cancer or in combination with other treatments such as surgery, radiation therapy, immunotherapy, or targeted therapy. There are various types of chemotherapy drugs used in cancer treatment. These drugs are classified based on their chemical structure, how they work against cancer cells, and their specific targets within the cells.

Some common types of chemotherapy drug include

Chemotherapy (chemo) is treatment with drugs to destroy cancer cells. Chemo may be an option for people whose liver cancer cannot be treated with surgery, has not responded to local therapies such as ablation or embolization, or when targeted therapy is no longer helpful. Most chemo drugs do not have a great effect on liver cancer. Recent advances have shown that a combination of drugs may be more helpful than using just a single chemo drug (*Zhang et al., 2020*). But even these combinations of drugs shrink only a small number of tumours, and the responses often do not last long. And most studies show systemic chemo has not helped patients live longer.

Alkylating agents: These drugs work by directly damaging the DNA of cancer cells, preventing their ability to replicate. Examples include cyclophosphamide, cisplatin, and carmustine (*Weber, 2014*).

Antimetabolites: These drugs interfere with the DNA and RNA production in cancer cells, disrupting their ability to grow and divide. Examples include methotrexate, 5-fluorouracil (5-FU), and gemcitabine (*Sun et al., 2021*).

Anthracyclines: These drugs block an enzyme necessary for DNA replication and can also generate free radicals that damage cancer cells. Examples include doxorubicin and epirubicin (*Yang et al., 2014*).

Topoisomerase inhibitors: These drugs interfere with enzymes called topoisomerases, essential for DNA replication. They prevent the DNA from repairing itself after normal cell division. Examples include etoposide and irinotecan (*Buzun et al., 2020*).

Mitotic inhibitors: These drugs disrupt the process of mitosis (cell division) in cancer cells by affecting microtubules, structures necessary for cell division. Examples include paclitaxel, docetaxel, and vinblastine (*Mukhtar et al., 2014*).

Hormone therapy drugs: These drugs interfere with the hormone signaling that some cancers use to grow. They are commonly used in hormone-sensitive cancers like breast and prostate cancer. Examples include tamoxifen and aromatase inhibitors (e.g., anastrozole, letrozole) (*Chaput and Sumar, 2022*).

Targeted therapy drugs: These drugs are designed to specifically target certain molecules or pathways involved in cancer cell growth. They can be more precise in targeting cancer cells while causing fewer side effects to healthy cells. Examples include trastuzumab (Herceptin), imatinib (Gleevec), and bevacizumab (Avastin) (Min and Lee, 2022).

Immunotherapy drugs: Immunotherapy drugs work by helping the immune system recognize and attack cancer cells. They include checkpoint inhibitors (e.g., pembrolizumab, nivolumab), CAR-T cell therapy, and monoclonal antibodies (Shiravand et al., 2022).

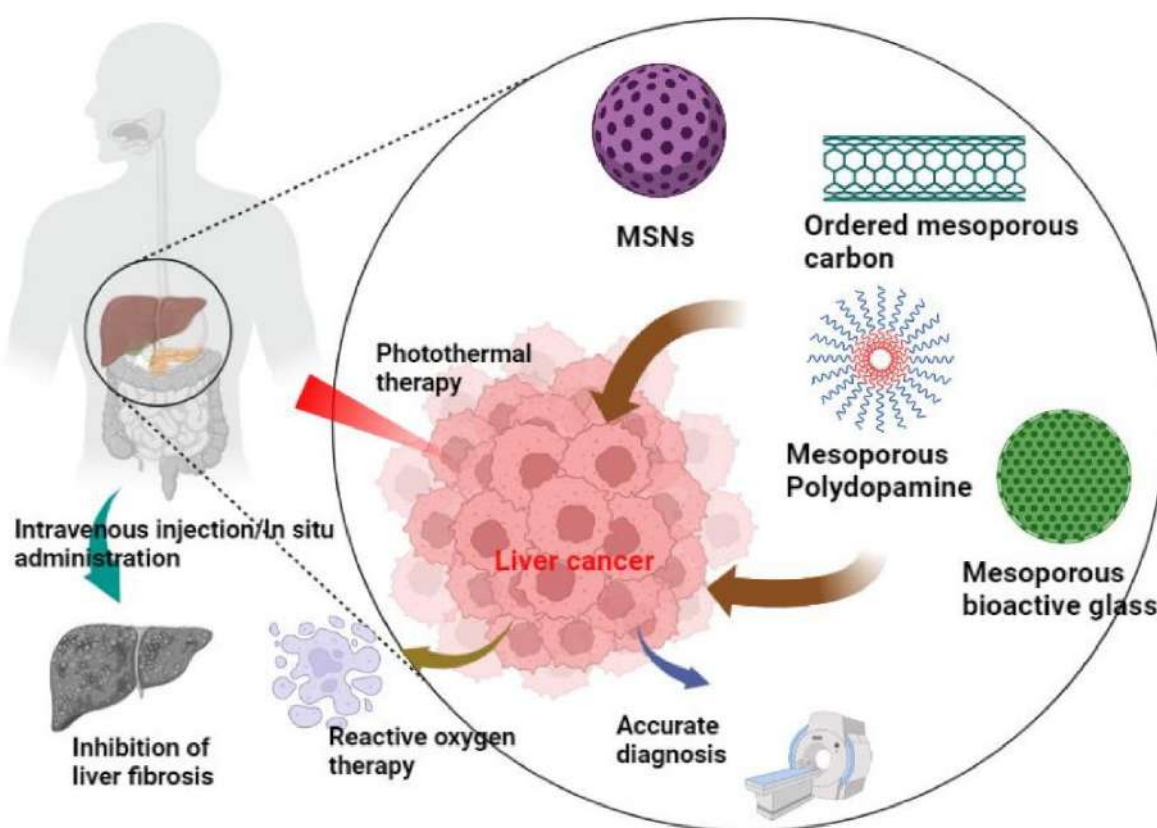


Fig:1.13 Summary scheme of mesoporous materials for the diagnosis and treatment of liver cancer. Currently, the types of mesoporous materials applied in the field of liver cancer mainly include mesoporous silicon nanoparticles, ordered mesoporous carbon, mesoporous polydopamine, mesoporous bioactive glass, etc. The biological applications of mesoporous materials include accurate diagnosis of liver cancer, systematic and local treatment and the treatment of cirrhosis (Wu et al., 2022).

1.6 Monacolin:

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, there have been studies examining its potential cytotoxic effects in various in vitro and in vivo models. In in vitro model Lovastatin has been found to induce apoptosis (programmed cell death) in certain cancer cell lines, including breast cancer and leukemia cells (*Cicero et al., 2019*). This suggests a cytotoxic effect on cancer cells. Monacolin has been shown to arrest the cell cycle at various stages in different cell types. This can lead to reduced cell proliferation and, in some cases, cytotoxicity. In animal models of cancer, monacolin has demonstrated potential tumour-suppressive effects. It may slow down tumour growth and reduce the risk of metastasis, likely through mechanisms such as anti-angiogenesis and immune modulation (*Moloudi et al., 2023*).

1.7 Monacolin-x

Our earlier study investigated with monacolin-x a secondary metabolite isolated from fungi-NMK7 associated with marine sponge showed antiproliferative/cytotoxic, antimigratory, apoptotic inducing ability on different breast cancer and normal cell lines. However, there is no clear evidence on antiangiogenic activity. Therefore, in this study, we have evaluated the antiangiogenic activity of monacolin-x using *in vitro*, *in vivo* and (*Nagabhishek et al., 2019b*). Monacolin-x, a naturally occurring compound found in red yeast rice, has garnered attention for its potential therapeutic benefits beyond its well-known cholesterol-lowering effects. Recent research has begun to unveil its intriguing properties in combating cancer, opening avenues for further investigation into its role as a potential anticancer agent. Monacolin-x, also known as dihydromonacolin L, shares structural similarities with lovastatin, a statin medication widely used to lower cholesterol levels. Like lovastatin, monacolin-x inhibits the enzyme HMG-CoA reductase, which plays a crucial role in cholesterol biosynthesis. However, its effects extend beyond lipid regulation, encompassing potential anticancer properties.

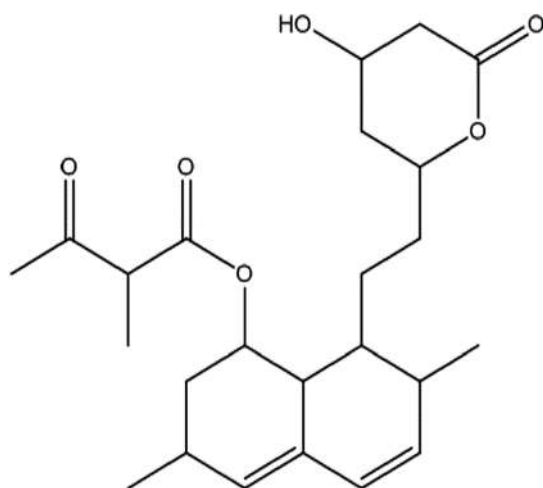


Fig:1.14 Structure of Monacolin-x.

1.7.1 Anticancer Mechanism:

Several studies have suggested that monacolin-x exhibits anticancer effects through various mechanisms, including:

Induction of Apoptosis: Monacolin-x has been shown to induce programmed cell death (apoptosis) in cancer cells, thereby inhibiting their proliferation and survival (Pistritto et al., 2016).

Anti-inflammatory Activity: Chronic inflammation is closely linked to cancer development and progression. Monacolin-x possesses anti-inflammatory properties, which may help mitigate inflammation-associated carcinogenesis (Singh et al., 2019).

Inhibition of Angiogenesis: Tumour growth and metastasis rely on angiogenesis, the formation of new blood vessels. Monacolin-x has demonstrated the ability to inhibit angiogenesis, potentially impeding tumour progression (Lugano et al., 2020).

Modulation of Signalling Pathways: Monacolin-x may interfere with various signalling pathways involved in cancer cell proliferation, differentiation, and survival, including the PI3K/AKT and MAPK pathways (He et al., 2021).

Enhancement of chemotherapy efficacy: Some studies suggest that monacolin-x may enhance the efficacy of chemotherapy drugs when used in combination. By

sensitizing cancer cells to chemotherapy, monacolin-x can help improve treatment outcomes and overcome drug resistance.

Antioxidant Properties: Monacolin-x may also possess antioxidant properties, which can help protect cells from oxidative stress and damage caused by free radicals. Antioxidants are important for overall health and may play a role in reducing the risk of chronic diseases such as cancer and cardiovascular disease (Pham-Huy et al., 2008).

Potential Cancer Prevention: While more research is needed, some studies have suggested that monacolin-x may have anticancer properties. It may inhibit the growth and proliferation of cancer cells, making it a potential candidate for cancer prevention and treatment. However, further studies are necessary to fully understand its effects on cancer (Hong et al., 2008).

1.7.2 Preclinical and Clinical Evidence

While preclinical studies have provided promising results regarding the anticancer potential of monacolin-x, clinical research in this area is still limited. Nonetheless, preliminary findings from both in vitro and animal studies have shown encouraging outcomes, warranting further investigation through well-designed clinical trials to validate its efficacy and safety in cancer treatment. Breast cancer is a complex disease with various subtypes and mechanisms of development. Research exploring the potential of lovastatin, specifically monacolin-x, in breast cancer is still in its early stages, but there are some promising findings and hypotheses. Preclinical studies have shown that lovastatin may possess anti-cancer properties. It has been observed to inhibit the growth of breast cancer cells in laboratory settings. Lovastatin appears to induce apoptosis (programmed cell death) and inhibit cell proliferation in breast cancer cell lines. Lovastatin functions by inhibiting HMG-CoA reductase, an enzyme involved in the mevalonate pathway, which is crucial for the production of cholesterol and other metabolites. This inhibition not only lowers cholesterol levels but also affects the synthesis of other important molecules such as isoprenoids. Disruption of the mevalonate pathway can interfere with cell signalling pathways involved in cancer progression. Chronic inflammation is associated with the development and progression of cancer, including breast cancer. Lovastatin has been shown to exhibit anti-inflammatory

effects by reducing the production of pro-inflammatory cytokines and inhibiting inflammatory pathways. This may contribute to its potential anti-cancer effects. There is also interest in exploring the potential synergistic effects of lovastatin when used in combination with other cancer treatments such as chemotherapy or targeted therapies. Some studies suggest that lovastatin may enhance the efficacy of certain anti-cancer drugs and reduce resistance to treatment. While much of the evidence supporting the anti-cancer effects of lovastatin comes from preclinical studies and observational data, there is ongoing interest in conducting clinical trials to evaluate its effectiveness and safety in breast cancer patients. These studies would help to clarify its potential role as an adjunctive or standalone therapy in breast cancer management.

1.7.3 Challenges and Future Direction

Despite its potential, several challenges must be addressed before monacolin X can be integrated into mainstream cancer therapy. These include elucidating its precise mechanisms of action, optimizing dosage regimens, assessing potential side effects, and conducting rigorous clinical trials to evaluate its efficacy in different cancer types and patient populations (*Chehelgerdi et al., 2023*). There is genetic variability in how individuals respond to lovastatin, affecting its efficacy and side effect profile. Personalized medicine approaches, such as genetic testing, could help optimize lovastatin therapy. Compliance and Adherence: Adherence to statin therapy can be challenging for some patients due to factors such as cost, inconvenience, and concerns about side effects. Developing strategies to improve patient compliance and adherence is crucial for maximizing the benefits of lovastatin. With the growing interest in natural and alternative therapies, there is ongoing research into other cholesterol-lowering agents derived from natural sources or through synthetic means. Exploring alternative therapies with potentially fewer side effects or better efficacy profiles could impact the future use of lovastatin. Developing novel drug delivery systems could improve the bioavailability and pharmacokinetics of lovastatin, enhancing its therapeutic effects while reducing side effects. Combination therapies involving lovastatin and other cholesterol-lowering agents or drugs targeting different pathways involved in cardiovascular diseases could offer synergistic effects and better outcomes for patients. Beyond its cholesterol-lowering effects, there is growing interest in

exploring potential additional therapeutic benefits of lovastatin, such as its anti-inflammatory, antioxidant, and neuroprotective properties. Further research in these areas could uncover new indications for lovastatin therapy. Regulatory requirements and safety considerations pose challenges for the development and approval of new formulations or indications for lovastatin. Overcoming these regulatory hurdles is essential for bringing innovative lovastatin-based therapies to market. Cost-effectiveness is an important consideration in healthcare decision-making. Assessing the cost-effectiveness of lovastatin therapy compared to other cholesterol-lowering interventions and optimizing healthcare policies accordingly is crucial for ensuring access to affordable treatment options.

1.7.4 Chemical Properties of Monacolin-x

Monacolin-x, also known as mevinolin or compactin, is a naturally occurring compound that belongs to the class of statins, specifically lovastatin (monacolin K). The chemical structure of the Monacolin-x has a lactone ring structure and is structurally similar to HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A), a precursor in the biosynthesis of cholesterol. The molecular formula of monacolin-x is $C_{24}H_{36}O_5$. Monacolin-x is sparingly soluble in water but dissolves well in organic solvents such as ethanol, methanol, and chloroform. Monacolin-x, like other statins, inhibits the enzyme HMG-CoA reductase, which is involved in the synthesis of cholesterol in the body. By inhibiting this enzyme, it reduces the production of cholesterol, which is beneficial for managing high cholesterol levels. Its stability can vary depending on environmental factors like temperature, light, and storage conditions. Due to its similarity to the pharmaceutical drug lovastatin, monacolin-x might be subject to regulatory scrutiny and restrictions in some regions when present in dietary supplements or natural products. Monacolin-x, when consumed, is absorbed in the gastrointestinal tract and undergoes metabolism in the liver, where it exerts its cholesterol-lowering effects.

1.7.5 Physical Properties of Monacolin-x

The physical properties of Monacolin-x, also known as mevinolin or compactin, include the appearance of the Monacolin-x is commonly found as a white to off-white crystalline powder or solid substance. The reported melting point for Monacolin-x is around 165-168°C (329-334°F). It is sparingly soluble in water but more soluble in organic solvents such as ethanol, methanol, and chloroform. The

molecular weight of Monacolin-x is approximately 404.54 g/mol. Typically, Monacolin-x is odourless and tasteless in its pure form. Its stability can vary depending on environmental factors like temperature, light exposure, and storage conditions. It is important to store it properly to maintain its integrity.

CHAPTER -2

LITERATURE SURVEY

2.1 Cancer

With 410 million deaths from cancer worldwide each year, it is one of the main causes of mortality. Current cancer treatments include surgery, radiation, and the use of chemotherapeutic medications, which frequently cause toxicity in patients and the death of healthy cells. So, scientists are trying to figure out how to only get rid of malignant cells. (*Hausman, 2019*) in his study proposed that the primary barrier to an effective cancer treatment is the heterogeneity of malignant cells within the tumour. The treatment of different malignancies can be enhanced, nevertheless, by a greater understanding of the molecular basis of tumours and the development of novel diagnostic technologies. Therefore, it is crucial that we investigate epigenetic modifications, the gene expression of malignant cells, and use techniques that allow us to reverse or lessen these changes. As Anya Plutynski puts it in *Explaining Cancer* (2018): "Cancer may be viewed as akin to a car breaking down, a process of natural selection, an infectious disease . . . a process of development, and even the growth of an ecological community". The most challenging characteristics of cancer metastasis, recurrence, and therapeutic resistance have recently been the subject of a convergence of theories that has produced a cohesive theoretical framework. The new paradigm of cellular differentiation, which was previously thought to be a unidirectional process but is now understood to be a flexible process in which cancer cells can dedifferentiate into more primitive, stem-like phenotypes, forms the core of this framework. Both inherent biochemical mechanisms and reciprocal environmental cues involving non- cancerous cells connected to cancer regulate this plasticity. Such flexible phenotypic changes may have an impact on malignancies' discontinuous behaviour, in which some tumours go untreated for months or even years before relapsing and wreaking havoc. (*Wael M et.al 2013*) also said that metastasis, relapse, and therapy resistance are three of the most difficult elements of cancer. The new paradigm of cellular differentiation, which was previously thought to be a unidirectional process but is now understood to be a flexible process in which cancer cells can dedifferentiate into more primitive, stem-like phenotypes, forms

the core of this framework. Both inherent biochemical mechanisms and reciprocal environmental cues involving non-cancerous cells linked to cancer regulate this plasticity. Such flexible phenotypic changes may have an impact on malignancies' discontinuous behaviour, in which some tumours go untreated for months or even years before relapsing and wreaking havoc. Somatic cells in the human body with genetic mutations accumulate to form cancers. (*Marc J et al 2019*) proposed that these mutations alter the cells' phenotype, enabling them to evade the homeostatic control that preserves a normal cell count. According to evolutionary biology, the conversion of healthy cells into cancerous cells is an example of evolution at action. Evolution caused by adaptation to changes in the cancer's environment, persists throughout cancer growth, progression, treatment resistance, and disease relapse, and intratumor heterogeneity is an inevitable byproduct of this evolutionary process. The use of genomics to characterise tumour evolution allows for the quantitative measurement of evolving clones over both space and time.

2.2 BREAST CANCER

Breast cancer remains a serious health issue despite significant advancements in the field of cancer research, and is currently a high focus for biomedical research. (*Zoi Anastasiadi et al,2016*) said the most frequent cancer among women worldwide is breast cancer, and both its incidence and fatality rates are predicted to rise sharply in the coming years. Recent cases of breast cancer in young women have caught the attention of researchers. According to recent data, breast cancer is clearly the top cause of cancer-related fatalities in women under the age of 45. The basic characteristics of this form of cancer appear to be potentially aggressive and highly heterogeneous. The 'complex' biology of this particular cancer type is still unknown and understudied, and therapy techniques, guidelines, and options are not based on age. (*Shai Libson et al 2014*), Breast cancer detection and diagnosis abilities have significantly increased. The underlying biological mechanisms are now being more heavily considered in treatment decisions that were formerly mostly centred on the anatomic extent of the disease. Breast cancer is now understood to be a heterogeneous disease made up of various biological processes thanks to the development of gene array technology. The ability to predict chemotherapy response is made possible by subtypes and genetic profiling. The oncoplastic method allows for broad excisions without

affecting the breast's natural contour, and breast conservation has become the accepted standard of treatment. The conventional approach to stage the axilla has been superseded by sentinel lymph node biopsy, sparing many patients the further morbidity of axillary dissection. (Kolak A et al 2017) said High-fat diets, alcohol use, and a lack of physical activity are just a few examples of lifestyle and environmental factors that have been shown to influence the development of mammary gland cancer; eliminating these factors (primary prevention) may help reduce morbidity and death. second line of defence, Including diagnostic tools that aid in the early diagnosis of cancers or lesions predisposing to tumours (such as mammography, ultrasonography, magnetic resonance imaging, breast self-examination, and contemporary, more accurate imaging techniques).

2.3 LIVER CANCER

Liver cancer could be broadly subdivided into hepatocellular carcinoma (HCC) (approximately 75%–85%), intrahepatic cholangiocarcinoma, as well as other mixed types according to the pathological type. It ranked as the sixth most common cancer and the third preponderant cause of cancer-related deaths worldwide. The distribution of liver cancer presented significant geographic disparities, and China contributed to nearly half of the global cases alone. Given the heavy burden of liver cancer at that time, rational approaches focusing on primary prevention and surveillance had been developed. In addition, a series of policies had been enacted by the central government to enlarge the coverage of hepatitis B vaccination. These measures had shown their efficacy in preventing the development of liver cancer and decreasing overall mortality. The epidemiology of liver cancer had been reported by domestic and international organizations, and the relevant healthcare policies and intervention measures had also been conducted based on the epidemiological data in China in the last decades. However, the related sources of information were wide-ranging and lacked comparisons. Thus, in this review, we aimed to describe the most recent patterns of liver cancer globally and in China, and then explore the major risk factors of liver cancer and the prevention measures conducted in China. An estimated 905,677 people were diagnosed with liver cancer worldwide in 2020 with an age-standardized incidence rate of 9.5 per 100,000. The age-standardized incidence rate of liver cancer was 14.1 per 100,000 for males and 5.2 per 100,000 for females. The liver cancer burden had dramatic geographic variations, which

was most pronounced in transitioning regions. The highest incidence of liver cancer was observed in Eastern Asia with an age-standardized rate of 17.8 per 100,000, followed by Northern Africa (15.2 per 100,000). In contrast, the lowest incidence rates in the world occurred in South-Central Asia (3.0 per 100,000), Central and Eastern Europe (4.3 per 100,000), and South America (4.3 per 100,000). Northern America had an intermediate level of incidence rate (6.8 per 100,000). However, the incidence of liver cancer in several regions of Europe and the United States gradually presented increasing trends. In 2016, liver cancer was the fourth most common cancer in China after lung cancer, colorectal cancer, and gastric cancer. An estimated 388,800 cases were diagnosed with liver cancer according to the most updated data from the National Cancer Centre with a crude incidence rate of 28.1 per 100,000 and an age-standardized incidence rate (using Segi's world standard population in 2000) of 17.7 per 100,000. About 74.3% (or about 288,800) of the reported cases were males, which was the third most prevalent malignancy in males. In females, liver cancer was the seventh most diagnosed cancer. The age-standardized incidence rates also showed significant sex disparities, and males had more three folds than the rate of females (26.7 per 100,000 vs. 8.7 per 100,000).

2.3.1 Epidemiology

Hepatocellular carcinoma scored second in the world for cancer-related deaths in 2005, making it the sixth most frequent type of cancer. Chronic viral hepatitis B and C infections were the primary cause of most occurrences of hepatocellular cancer. The Surveillance, Epidemiology and End Results (SEER) Database for the United States revealed a 3.1% annual rise in incidence. The incidence was 11.5 per 100,000 for men and 3.9 for women. Additionally, the annual death rates from hepatocellular carcinoma had risen by 2.8% for men and 3.4% for women. Hepatocellular carcinoma typically manifests in the elderly following a protracted chronic liver illness. The exposure risk factor influenced the hepatocellular carcinoma variances by region and race/ethnicity. Globally, HBV was more common, and in the US, HCV accounted for 30% of cases. Between 1945 and 1965, the frequency of HCV grew fivefold in Americans born in the country, increasing the risk of liver cancer.

2.4 MONACOLIN

China has been using *Monascus* fermentation products for culinary and medicinal purposes for over a millennium, particularly those derived from the solid-state fermentation of rice. In the United States and several Asian nations, including Japan, Taiwan, China, Korea, Thailand, the Philippines, and Indonesia, monascus rice products, or MRPs, are currently consumed as health foods. Numerous research investigations have demonstrated that *Monascus* species produce food colorants, cholesterol-lowering drugs, and antibiotics that are commercially viable. The most significant bioactive substance discovered in *Monascus* is monacolin K, which is the same as the powerful anti atherosclerotic, cholesterol-lowering medication lovastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor. In addition, a number of *Monascus* species generate citrinin, a mycotoxin that is detrimental to the kidneys and liver. Two polyketide fungi metabolites are citrinin and monacolin-k. The biosynthetic routes of *Aspergillus* and *Monascus* have been shown to produce polyketides, such as citrinin and monacolin K. Therefore, there is a great deal of concern about safety while developing MRPs as health foods. Recent research reveals that MRPs contain additional chemicals (flavonoids, polyunsaturated lipids, phytosterols, pyrrolic compounds, and others) with a wide range of biological activities and pharmacological potentials, suggesting that MRPs may have additional useful applications. They have stronger effects than monacolin K alone in decreasing blood sugar and triacylglycerol and boosting HDL-C. In addition to reducing cholesterol, MRP might be a great option for treating metabolic syndrome (*Wang and Lin, 2007*).

2.5 MONACOLIN X

With 719 citations (701 for the period January to December 2019) referring to compounds isolated from marine microorganisms and phytoplankton, green, brown, and red algae, sponges, cnidarians, bryozoans, molluscs, tunicates, echinoderms, mangroves, and other intertidal plants and microorganisms, this review covers the literature published in 2019 for marine natural products (MNPs). The focus is on novel compounds (1490 in 440 publications for 2019), together with the biological activity, source organisms, and nation of origin that are

pertinent. Included are important reviews, biosynthetic investigations, first syntheses, and syntheses that resulted in structural or stereochemical revisions. Techniques for investigating the chemical diversity of marine fungus have also been covered (Nagabhishek *et al.*, 2019b).

2.5.1 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 frondi fera* was isolated by (Nagabhishek and Madankumar, 2019) and the isolated metabolite with molecular weight of 418.5 Da and molecular formula C₂₄H₃₄O₆, 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 signalling 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.

2.6 Role of IL-6 in liver cancer

The majority of cases have underlying chronic inflammatory liver disorders. The growth and progression of HCC may be positively or negatively impacted by inflammatory cytokines. As a significant pro-inflammatory mediator, interleukin-6 (IL-6) is a strong mitogen for cells and cholangiocarcinoma cells. Concurrently, IL-6 is recognized as an autocrine growth factor in the field of cancer (Shakiba *et al.*, 2018). IL-6 appears to stimulate a mitogen-activated protein kinase signalling pathway, which in turn increases the proliferation of cholangiocarcinoma cells.

Higher Child-Pugh scores, greater tumour sizes, low platelet counts, liver cirrhosis, elevated α -fetoprotein (AFP) levels, and high serum hepatitis B virus (HBV) loads are significant risk factors of recurrence in successfully treated early-stage HCC patients (*Myojin et al., 2022*).

2.7 Role of IL-1 β in liver cancer

Chronic inflammation is caused by the activation of resident immune cells and the migration of additional leucocytes to the injured liver, which is facilitated by the liver's production of IL-1 β , pro-inflammatory IL-6, and TNF α . Therefore, as evidenced by lower serum transaminases and serum amyloid alpha concentrations, as well as decreased expression of inflammatory and fibrosis genes, IL-1 β deficient animals have shown less diet-induced inflammation and liver fibrosis (*Rebe and Ghiringhelli, 2020*). In the adipose tissue of obese and insulin-resistant mice, there is an upregulation of IL-1 β . In conjunction with TNF α and IL-6, IL-1 β also plays a role in the development of insulin resistance in adipocytes by suppressing the production of insulin receptor substrate-1, which in turn promotes the buildup of lipids in the liver (*Barbier et al., 2019*).

2.8 Role of TNF- α in liver cancer

Hepatocellular carcinoma (HCC) is an inflammation-related disease. Tumour necrosis factor alpha (TNF- α) is an important inflammatory factor and it has been confirmed to promote tumour growth and poor prognosis of HCC. Hepatic progenitor cells (HPCs) are thought to play an important role in liver injury and repair, as well as tumorigenesis. Tumour necrosis factor- α (TNF α) is an inflammatory cytokine involved in liver inflammation and sustained liver inflammation leads to liver fibrosis. TNF α exerts inflammation, proliferation, and apoptosis. However, the role of TNF α signalling in liver fibrosis is not fully understood. This review highlights the recent findings demonstrating the molecular mechanisms of TNF α and its downstream signalling in liver fibrosis. During the progression of liver fibrosis, hepatic stellate cells play a pivotal role in a dynamic process of production of extracellular matrix proteins and modulation of immune response (*Yang and Seki, 2015*).

2.9 Role of Bax and Bcl-2 in liver cancer

The purpose of this study was to determine the frequency of expression of apoptotic markers in hepatocellular carcinoma (HCC) and their relationship with prognosis. Seventy HCC were immune stained for bcl-2, bax, Staining intensity in tumour cells was graded 0 to 3+. Follow-up data were available for mean survival (57 cases) and death rates (58 cases). These values and clinical parameters were related to prognosis. Staining frequency for bcl-2, bax, was 20% and 60%, respectively. Immunostaining intensity of bax correlated with overall survival and death rates: of 57 patients, the 37% with 0 to 1+ intensity had a median survival of 6.6 months, the 63% with 2 to 3+ intensity had a median survival of 31.9 months ($P = 0.05$); 86% of 19 patients with 0 to 1+ intensity died, and 50% of 36 patients with 2 to 3+ intensity died ($P < 0.05$). There was no correlation between bcl-2 expression, stage, or gender and prognosis. Patients with bax expressing HCC experience improved survival compared with those with no or low bax expression, in uni and multivariate models. Bcl-2 expression did not correlate with prognosis (Garcia et al., 2002).

CHAPTER-3

AIM AND OBJECTIVES

3.1 Aim

The main aim of the study is to check the anticancer effect of monacolin-x against Human Liver cancer cell line.

3.2 Objectives

- The cytotoxic effect of monacolin-x against zebrafish embryos for the developmental deformities.
- To determine the optimal dosage range and treatment regimen for Monacolin-x.
- To develop a novel and effective targeted treatment strategy for Liver cancer.
- To Evaluate whether the Apoptosis Mechanism of liver cancer cells is affected by Monacolin-x.
- Find out if Monacolin-x has any anti-metastatic effects on cells that cause liver cancer.
- To explore the potential role of monacolin-x on inhibition of liver tumor (HepG2 cells) growth.
 - Apoptosis assay
 - MTT assay
 - Gene expression analysis of apoptosis
 - Apoptosis cell staining (Ao/EB and PI)

CHAPTER - 4

MATERIALS AND METHODS

4.1 Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM) (Himedia; Cat log-AL007S#....), Fetal Bovine Serum (FBS) (Cat log-RM10432), 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Cat log-33611), Dimethyl sulfoxide (DMSO (Cat log-43404), Propidium Iodide (PI (Cat log-TC252), Acridine Orange (AO) (Cat log-TC262), and Ethidium bromide (EB) (Cat log-RM813), were purchased from hi-media (California, USA). All other reagents were purchased from Sigma Aldrich (USA) and were of analytical grade.

4.2 *Invitro* Cell line Studies

In vitro human cell line models are commonly utilized in cancer pharmacogenomic investigations to find novel pathways linked with variance in drug response, to assist establish pharmacogenomic hypotheses for additional testing, and to predict clinical response. An example of an immortalized cell line model system is the HepG2 cell line, which is used to represent liver cancer in humans. The National Centre for Cell Science (NCCS), Pune, is where the human hepatocarcinoma (HepG2) cell line was obtained. Primarily, these models have been employed to ascertain how somatic changes impact the reaction to anticancer treatment as per our previous reports (Niu and Wang, 2015).

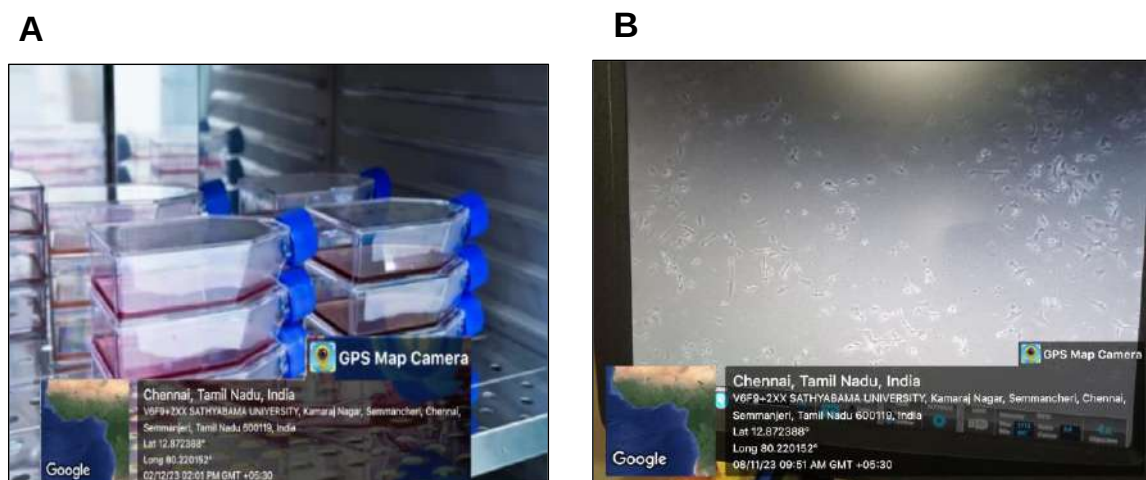


Fig:4.1 HepG2 cell line.

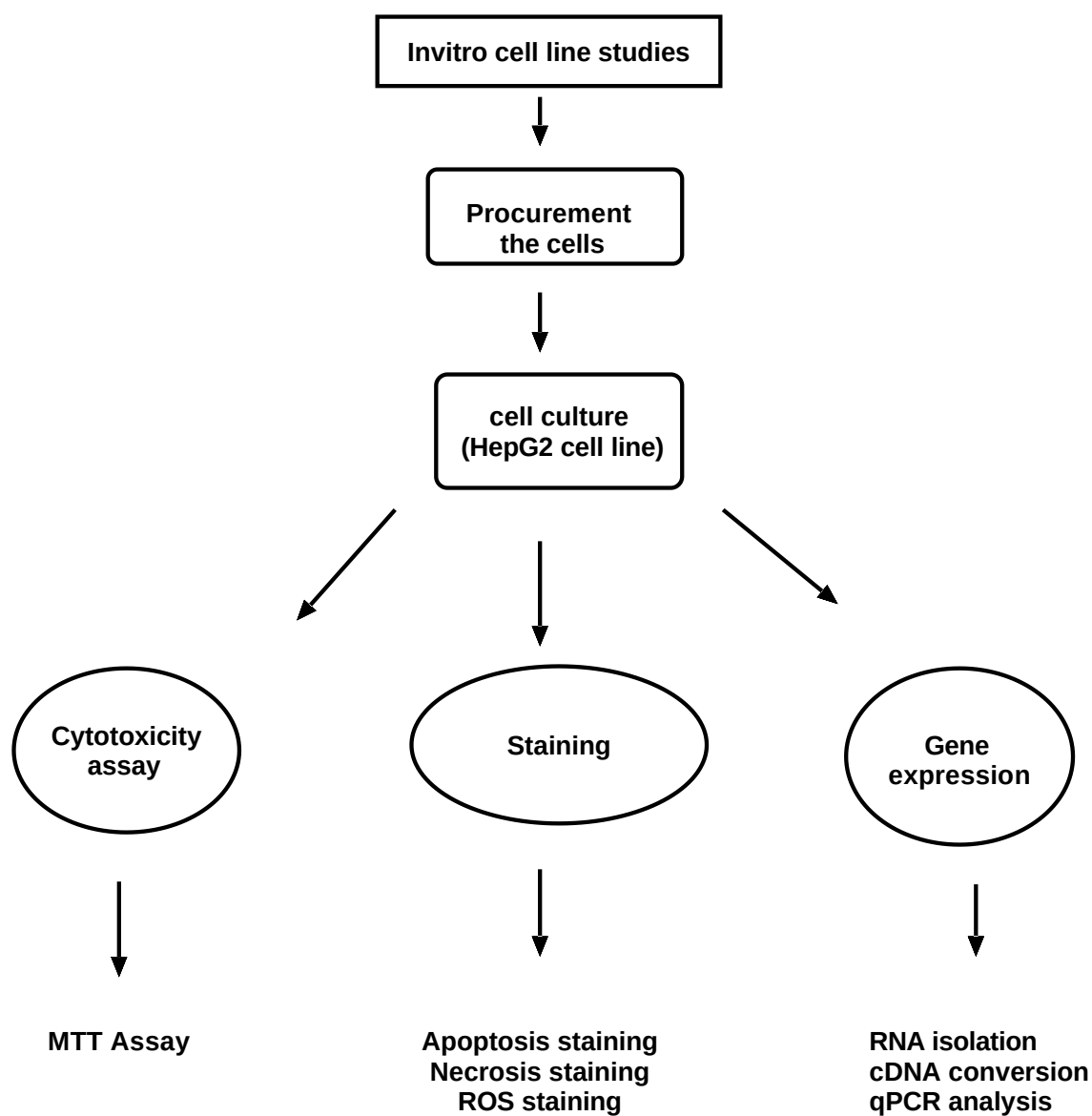
4.2.1 Cell line Maintenance

The cell line were maintained in DMEM medium supplemented with 10% FBS containing 100 U/mL penicillin, and 100µg/mL streptomycin. Cells were grown in T25 flasks at 37 °C in a humidified atmosphere containing 5% CO₂. In a culture vessel, the cells are grown to confluence, generating a monolayer that adheres to the surface. Next, additional cells are sub cultured by the use of the trypsinization process. The culture medium is then aspirated, and 2ml of phosphate buffered saline (PBS) are used to rinse the cells in order to get rid of any remaining medium and cellular debris. Add 1 ml of 0.05%trypsin, and let it trypsinate for three to five minutes at 37°C. To see cells separate, gently tap the side of the dish; if this is not done carefully, over trypsinization may happen. To neutralize the trypsin, 2 ml of medium is then added. After three minutes of room temperature spun at 900 rpm, the cell suspension is aspirated to remove the supernatant. After collection, the pellet was reconstituted in 2 ml of DMEM medium containing 10% FBS. After that, the cells were divided into four sterile, clean bottles, each holding a volume of 0.5ml. The bottles were then filled to a capacity of 2 ml with DMEM media.

Cell line	Media used	Source of cell line
Fibroblast cell line	DMEM with 10% FBS	National Centre for Cell Science (NCCS), Pune
Liver cancer cell line	DMEM with 10% FBS	National Centre for Cell Science (NCCS), Pune

Table 4.1 List of Cell Lines was used

4.2.2 Experimental design



4.2.3 Drug preparation

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

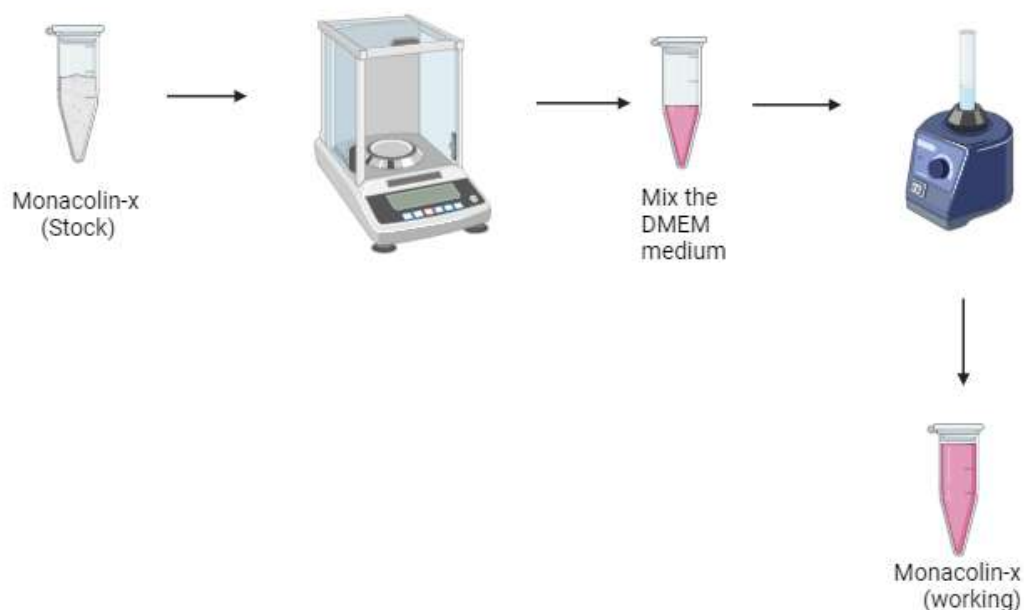


Fig:4.2 Schematic representation of monacolin-x

4.2.4 Cytotoxicity Assay

The cell viability have been measured using MTT assay. The MTT assay for the quantitation of growth modulating effects on cultured Liver cancer cell lines was investigated. Schematic shown in (fig:4.3). Liver cancer cells (HepG2) and normal liver cells were seeded into 96-well plates and were treated with different concentrations of extract for 24 h. After incubation, 50 μ L of MTT (1mg/ mL) was added to the cells and incubated at 37 °C for 4 h. The MTT insoluble formazan was dissolved in DMSO. The MTT reduction was quantified by measuring the absorbance at 570 nm The IC₅₀ value was defined as the concentration of extract that caused a 50% inhibition of cell growth compared to control cells.

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

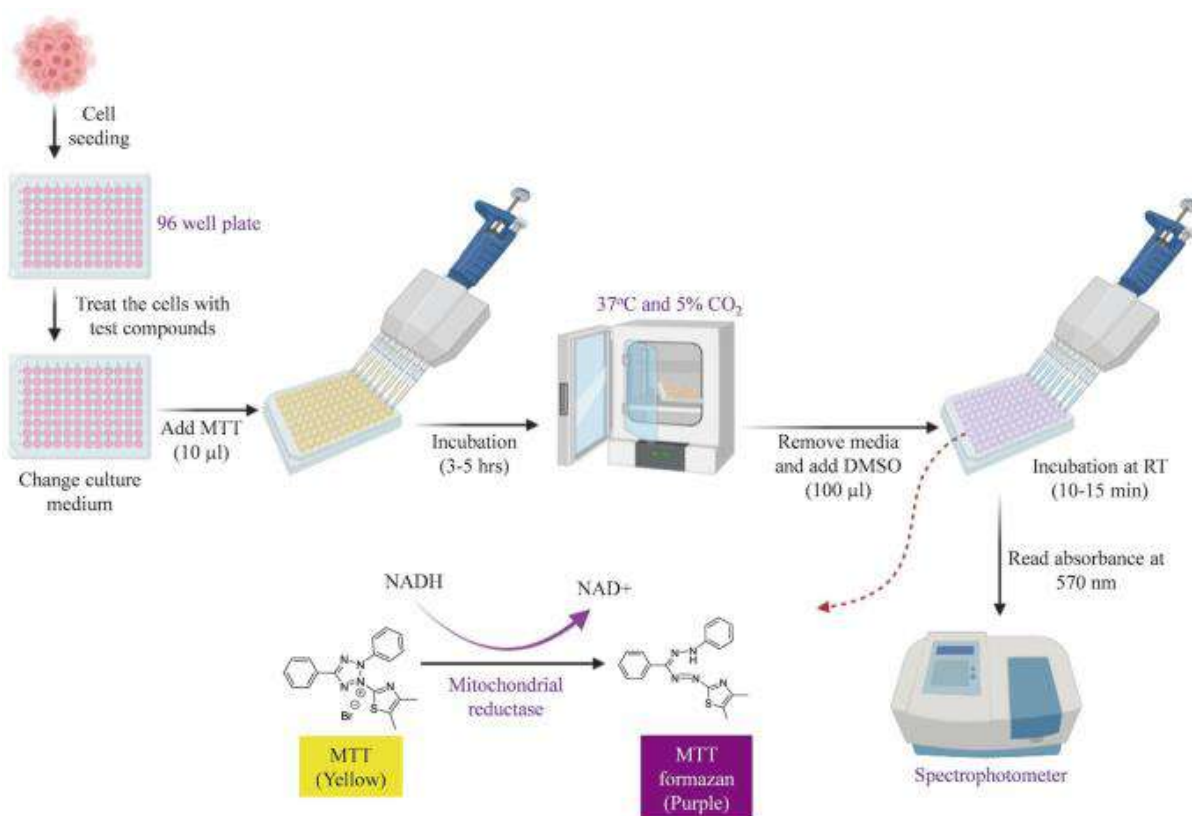


Fig:4.3 Cytotoxicity Assay

4.2.5 Necrosis assay by PI staining

PI is a viability dye. Immunofluorescence microscopic analysis method was used to assess cell necrosis. Immunofluorescence microscopic analysis of PI-stained cells provides a quantitative measure of the proportion of live and dead cells in a population by imaging. This assay measures the integrity of the cell membrane, which is compromised during necrosis. Techniques like (PI) or lactate dehydrogenase (LDH) release assays quantify the release of cellular components (such as LDH enzyme or PI dye) from damaged cells into the surrounding medium. Real-time imaging techniques like time-lapse microscopy can track changes in cell viability associated with necrosis.

4.2.6 Apoptosis assay

The cell toxicity was identified with monacolin treatment based on the apoptosis induced properties. The apoptosis assay was performed with Acridine Orange/Ethidium bromide (AO/EB) staining. The cells should put well plates then, treated the drug from the cell and after many hours add the acridine orange and

ethidium bromide 2 μ l (1mg/ml) in plate. Incubated at room temperature for 15 minutes. Real-time imaging techniques like time-lapse microscopy can track changes in cell toxicity associated with apoptosis.

4.2.7 ROS Staining

In sterile DMSO, make a fresh stock solution of DCFDA just before using it. Steer clear of frozen and thawed dye several times. To eradicate any remnants of the initial medium, give the cells a wash in either phosphate-buffered saline (PBS). We utilized the human hepatocarcinoma cell line in this experiment. In standard culture media with decreased serum (2%), use DCFDA at a final concentration of 1 μ M. In an ordinary incubator set at 37°C with 5% CO₂, incubate the cultures for 30 minutes in the dark. Eliminate any leftover dye solutions. After removing the medium containing DCFDA, wash twice with PBS. Proceed with caution as you shield your cells from light. To the DCFDA-loaded cells, add fresh medium containing your preferred medication, and then continue to incubate as needed. We utilize H₂O₂ (0.03%) for one hour in this example. Analyse your cells right away using fluorescence microscopy, a fluorescence plate reader utilizing the FL1 channel (green fluorescence) to determine the level of ROS. Using excitation and emission wavelengths suitable for green fluorescence, the fluorescence can be observed.

4.2.8 DPPH Staining

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical compound commonly used in antioxidant assays to measure the antioxidant activity of substances. When DPPH reacts with an antioxidant compound, it is reduced, resulting in a colour change from purple to yellow, which can be measured spectrophotometrically. Prepare a stock solution of DPPH by dissolving it in a suitable solvent to a concentration. The exact concentration may vary depending on the sensitivity of the assay and the expected antioxidant activity of the samples. The DPPH solution should be freshly prepared as needed. Then, prepare the test sample. In a microplate or cuvette, mix the DPPH solution with an equal volume of each test sample or control antioxidant solution. Use appropriate controls, such as solvent blanks, to account for any background absorbance. Incubate the reaction mixture in the dark at room temperature or at a specified temperature for a defined period. The incubation time may vary

depending on the expected reaction kinetics and the antioxidant activity of the samples, but typically ranges from 15 minutes to 1 hour. After the incubation period, measure the absorbance of the reaction mixture at the appropriate wavelength (usually around 517 nm) using a microplate reader or spectrophotometer. Record the absorbance values for each sample and control.

4.2.9 RNA Isolation

Isolating RNA from liver cell involves specific considerations due to their small size and potential susceptibility to RNA degradation. Culture the liver cells and then the cells should keep on the well plate. Add the Monacolin-x in IC50 Concentration and incubate the 24 hours. The first step was aspirate the media and wash once with 2ml and aspirate the PBS. Remove the PBS as much as possible and add 1ml of Trizol. Scrape the cells from the plate briefly, then remove the Trizol with a pipette and deposit the Trizol into a 2ml Eppendorf tubes. Leave at room temperature for 5 minutes and add 250µl chloroform and shake the tube vigorously for about 15 seconds. Leave at room temperature for 5 minutes. Centrifuge the samples to separate the phases (aqueous, interphase, and organic). And then, Transfer the aqueous phase (containing RNA) to a new tube and add isopropanol to precipitate the RNA. Centrifuge the tube to pellet the RNA and Remove the supernatant and wash the RNA pellet with ethanol to eliminate impurities. Air-dry or speed-vacuum the RNA pellet to remove residual ethanol. Resuspend the RNA pellet in RNase-free water. Optionally, add an RNase inhibitor to prevent RNA degradation and Measure the RNA concentration and purity using a spectrophotometer.

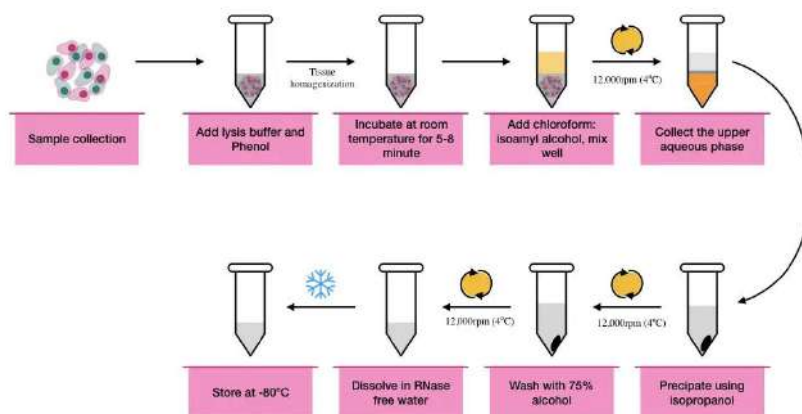


Fig:4.4 RNA ISOLTAION

4.2.10 RNA Quantification

It is common practice to quantify RNA by reverse transcription and then amp up nucleic acids using polymerase chain reaction (PCR). However, the quality of the RNA greatly influences the outcome. This could differ greatly since RNA can be broken down by a wide range of physical and chemical agents, such as tissue ribonucleases, heat, radiation, and chemicals. In the case of preserved tissue, fixed samples, and archival specimens, it could be challenging to prevent this. The quality of RNA has been evaluated using a variety of methods. However, it has been reported that improving the RIN algorithm can enhance RT-qPCR quantification, which leads to more quantitative inferences from the data. Nevertheless, these approaches only yield a qualitative or semi-quantitative estimate of RNA integrity. Although an analysis of the sample's integrity may be indicated qualitatively, a quantitative measure allows for the correction of RNA target measurement for degradation.

4.2.11 cDNA conversion

Isolating cDNA from a specific compound like monacolin-x involves a specific and meticulous process. The sample preparation of the CDNA Isolation is the extracting the monacolin-x from the source material (like red yeast rice) would be the initial step. This might involve extraction methods using solvents, chromatography, or other purification techniques and then once the compound is extracted, you would need to isolate RNA from the cells or tissues that produce monacolin-x. And Reverse transcription was usually done using specialized kits and involves breaking down the cell walls and membranes to release the RNA and the another step is using an enzyme called reverse transcriptase to convert the RNA into cDNA. This process is called reverse transcription and results in the creation of complementary DNA strands from the RNA template. Following reverse transcription, the cDNA needs to be purified to remove any remaining RNA, enzymes, or impurities. This can be done using various purification kits or techniques such as column purification, ethanol precipitation, or gel purification. After purification, the cDNA should be quantified to determine its concentration and verified for its quality using techniques like spectrophotometry or gel electrophoresis.

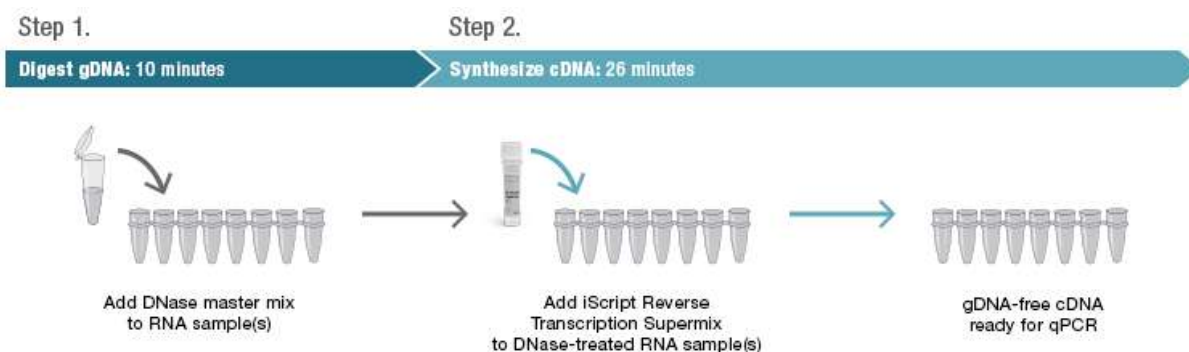


Fig:4.5 cDNA Conversion

4.2.12 Primer Designing

A primer is a brief single-stranded DNA fragment that is utilized in specific laboratory procedures, such the polymerase chain reaction (PCR), in the context of genomics. Through the use of two primers that hybridize with the sample DNA, the PCR process produces millions and millions of copies of the target DNA in a relatively short amount of time. DNA sequencing and other experimental procedures also make use of primers. We have to use inflammatory gene expression (IL-6, IL-1 β and TNF- α) and apoptosis gene expression (Bax and BCl-2) by qPCR analysis.

S.NO	TARGET GENE	GENE ID	FORWARD PRIMER (5' $\rightarrow$ 3')	REVERSE PRIMER (5' $\rightarrow$ 3')	AMPLICON LENGTH (bp)	Ta (°C)
1.	IL-6	3569	ATAACCACC CCTGACCC CAAC	ATACCTCAAA CTCCAAAAG ACC	149	55.1
2.	IL-1 β	7124	CTCTCACCT CTCCTACTC AC	ACACTGCTA CTTCTTGCC C	194	54.3
3.	TNF- α	3553	AACCATTCT CCTTCTCCC C	TTTCACCTCC ATCCATCCT C	186	51.3

Table.4.2 Inflammatory gene expression by qPCR analysis

S.NO	TARGET GENE	GENE ID	FORWARD PRIMER (5'→ 3')	REVERSE PRIMER (5'→ 3')	AMPLICON LENGTH (bp)	Ta (°C)
1.	Bax	12028	CTACAGGG TTTCATCC AG	CCAGTTCA TCTCCAA TTCG	118	54
2.	BCL-2	12028	GTGGATGA CTGAGTAC CT	CCAGGAGA AATCAAAC AGAG	133	56

Table.4.3 Apoptosis gene expression by qPCR analysis

4.2.13 RT-PCR Analysis

RT-PCR (reverse transcription-polymerase chain reaction) is a variant of the polymerase chain reaction (PCR) which are now widely used. Traditionally RT-PCR involves two steps: the RT reaction and PCR amplification. RNA is first reverse transcribed into cDNA using a reverse transcriptase as described here, the resulting cDNA is used as templates for subsequent PCR amplification using primers specific for one or more genes. RT-PCR can be used to quantify mRNA levels from much smaller samples. In fact, this technique is sensitive enough to enable quantitation of RNA from a single cell.

4.3 Toxicology Studies

Zebrafish (*Danio rerio*) has been extensively studied and well described for environmental toxicity studies. Molecular biology and genetics have recently been used to elucidate the underlying mechanisms of toxicity in zebrafish and to predict effects in mammals. The versatile zebrafish is now incorporated in many areas of toxicological programs for assessing human risk and for preclinical drug discovery and screening.

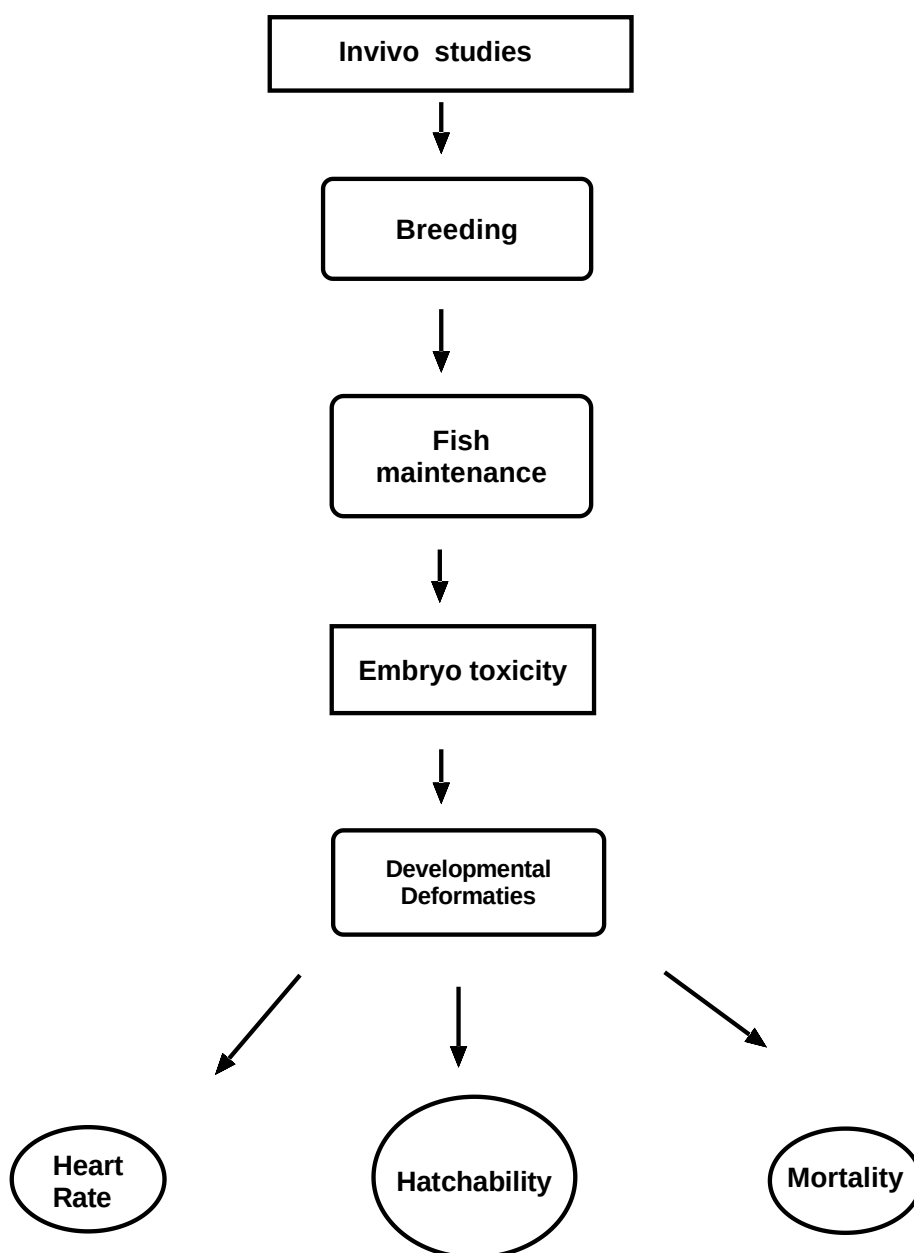
4.3.1 *In vivo* developmental toxic level of zebrafish using monacolin

The zebrafish (*Danio rerio*) embryo was used as an embryonic and larval model to perform *in vitro* experiments and developmental toxicity studies. Zebrafish embryos have been extensively used to study toxicological determinations of chemotherapeutic molecules by checking the mortality, hatchability, heart development and cardiac function, mainly due to the unique embryology and genetics of this model organism. Since most human disease occurs during adulthood, adult zebrafish models of heart disease are being created to dissect mechanisms of the disease and discover novel therapies (Hoage *et al.*, 2012). The zebrafish embryo was used in this study to establish the toxic effect of monacolin on early stage of development.

4.3.2 *Fish Maintenance*

The zebrafish were kept in cancer biology lab, international research centre, Sathyabama Institute of Science and Technology, Chennai. Zebrafish of wild-type (AB line) was used in this study were feed according to standard procedures (Karanam and Arumugam, 2022). Briefly, fish were kept with constant temperature at $28 \pm 0.5^{\circ}\text{C}$ and 14/10hour light/dark photoperiod in an aquarium. The fish were fed with live brine shrimp twice a day and dry flakes once a day. The pH value of the water varies from 7.2 to 7.4. Before mating, adult male and female zebrafish were separated in a breeding tank at the ratio of 2:1. After the light on the next morning, embryos were generated by natural mating. Then the eggs were collected, rinsed three times and cultured in incubator before subsequent experiments. The embryos were checked at 6hpf under the microscope to make sure all of the embryos stay at the same development stage. At 24hpf, embryos were examined under the microscope to make sure the lethal rate lower than 0.5%. Then the embryos can be used in the subsequent experiments.

4.3.3 Experimental design



4.3.4 Treatment of Zebrafish embryos with monacolin-x

The embryos at 4hpf were examined under the stereomicroscope to assure they were at the same developmental stage. Then 20 embryos were randomly distributed into six-well plates (20 for each concentration) and exposed to monacolin-x (0.5, 1.0, 1.5, 2.0 μ M) dissolved in 3 ml of the E3 medium for 0, 24, 48, 72 and 96 hours. The control group was only treated with E3 medium. The solutions were replaced and the dead embryos were removed at 24-hour interval. All tests were repeated three times. As described above, embryos at 4hpf exposed

to different concentrations of monacolin-x (0.5, 1.0, 1.5, 2.0 μ M) were recorded and photographed the phenotypic changes at different concentration (0, 24, 48, 72 and 96 hrs).

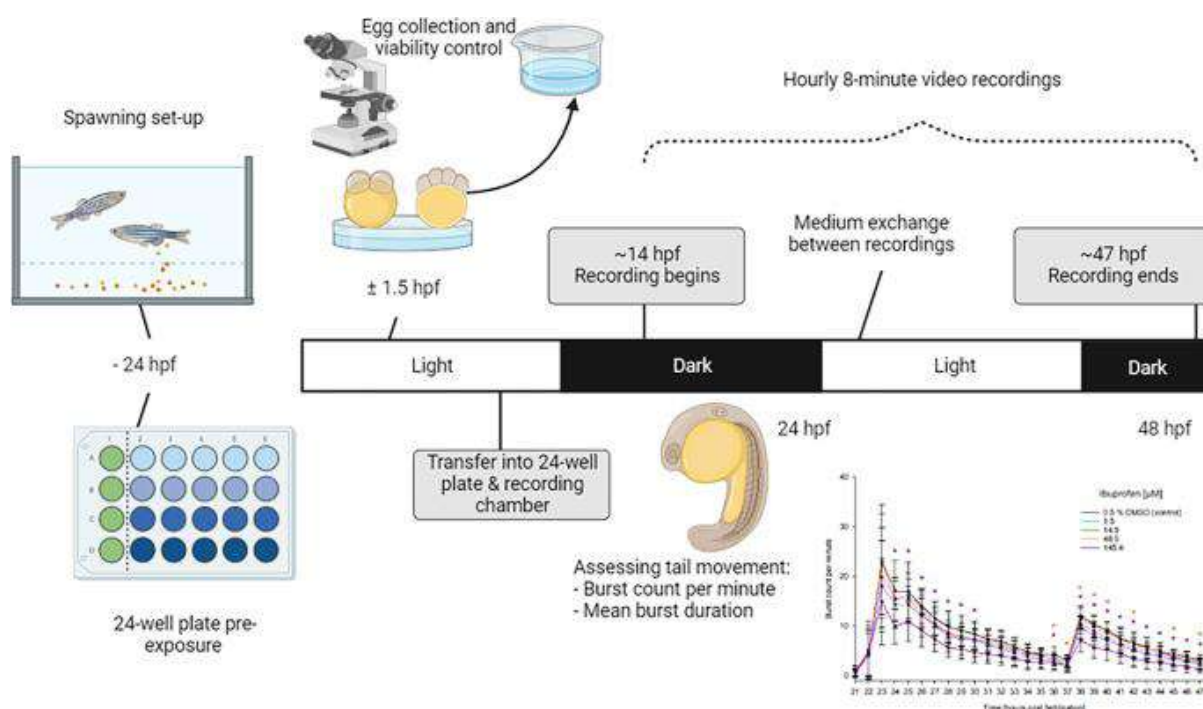


Fig:4.6 In vivo developmental toxic level of zebrafish using monacolin-x

4.3.5 Developmental deformities

Developmental deformities refer to structural abnormalities or irregularities that occur during the development of an organism. These deformities can arise at various stages of development, starting from embryonic growth to the fetal stage and even during childhood.

4.3.6 Heart Rate

After treatment with monacolin-x for 48 hours, the heart beating for 20 seconds was recorded, and the number was multiplied by 3 to calculate the heart rate. The area of peri cardiac edema and distance between the cardiac sinus venosus (SV) and bulbus arteriosus (BA) in the transgenic zebrafish were identified according to the stereo microscope analysis and used ImageJ program for the quantification. The experiments were conducted in three replicates and all tested were repeated for three times.

4.3.7 Hatchability

The hatching rate and the malformation rate at different time points was calculated and analysed (The hatching rate = the hatching embryo/total number of the embryo * 100%. The malformation rate = the number of the malformation embryo/the number of the embryos alive * 100%). The body length of the zebrafish larvae at 96hpf were measured and analysed using ImageJ program. In subsequent experiments, the concentrations were selected according to the malformation rate. One concentration is below 10% malformation rate and one between 10 and 50%, one is about 50% and one is more than 50%. Zebrafish embryos were divided in individual wells of the 6-well plate, with each well containing the E3 embryo medium. Different concentrations of monacolin-x were added in each wells, leaving some wells as controls. The zebrafish embryos were administrated the different time interval 0,24,48, 72 and 96 hours.

$$\text{The hatching rate (\%)} = \frac{\text{Number of hatching embryos}}{\text{Total No. of embryos}} \times 100$$

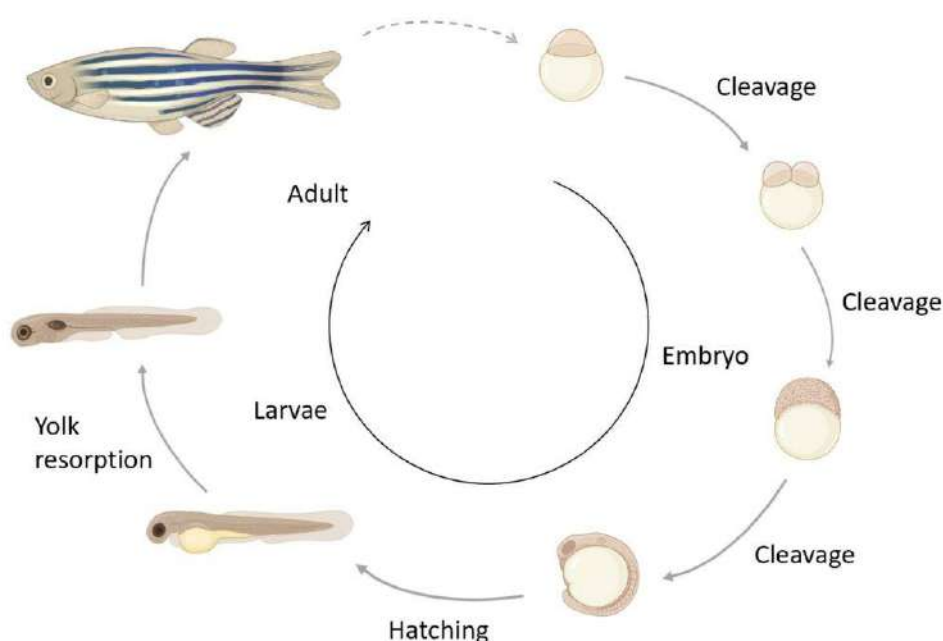


Fig:4.7 Zebrafish life cycle

4.3.8 Mortality

Zebrafish embryos can have genetic mutations that affect their development and viability. These mutations may result in embryonic lethality. Factors such as water

quality, temperature fluctuations, pH imbalance, and exposure to toxins can adversely affect zebrafish embryos and lead to mortality. Improper handling during embryo collection, manipulation, or transfer can cause physical damage or stress to the embryos, leading to mortality. Bacterial, viral, or fungal infections can infect zebrafish embryos, causing mortality. Proper sanitation and hygiene protocols are essential to prevent infections in zebrafish facilities. Inadequate nutrition in the form of improper feeding or nutrient imbalance in the water can lead to developmental abnormalities and mortality in zebrafish embryos. Some embryos may fail to develop properly due to developmental defects, which can result in mortality at various stages of development.

$$\text{The Mortality rate (\%)} = \frac{\text{Number of dead embryos}}{\text{The Total No. of embryos}} \times 100$$

CHAPTER-5

RESULTS AND DISCUSSION

5.1 *Invitro* anticancer studies

5.1.1 *Experimental design*

In this present study we aimed to explore the anticancer effect of monacolin-x against human liver cancer cell line (HepG2). The experimented groups were divided into two groups, in which group1 served as control liver cells (untreated) and group 2 serves as monacolin-x treated for 24hrs. Initial study, group 2 were fixed from the different concentrations of monacolin-x treatment and confirmed IC50 value.

5.1.2 *Drug preparation*

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

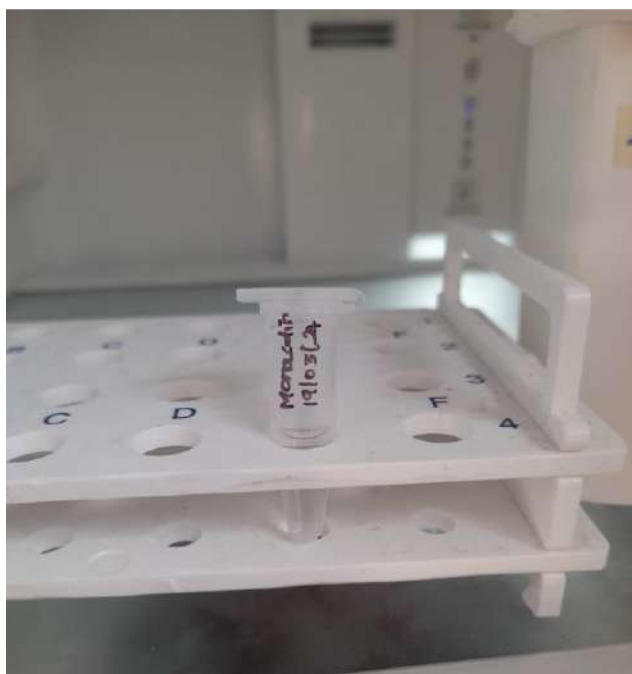


Fig:5.1 Dissolved Drug

5.1.3 Cytotoxicity assay

The control, monacolin-x were incubated individually with cancerous HepG2 cells and non-cancerous mouse Fibroblasts (3T3L1) cells to study the inhibition of the cell growth rate using MTT assay.



Fig:5.2 96 well plate, after adding the MTT.

The corresponding cell viability graph for different concentrations was shown in Fig.5.3-5.4 The absorption percentage of treated cells compared to control cells was used to calculate the percentage of cell viability.

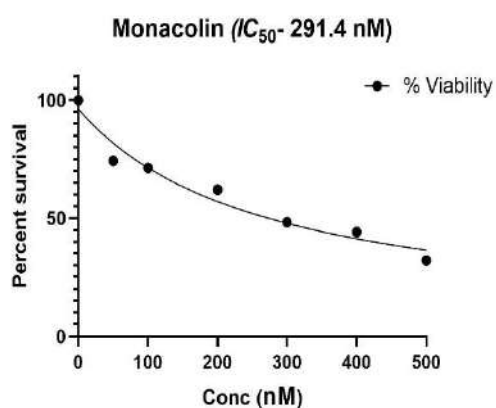


Fig:5.3 The MTT assay result of Fibroblast cell line.

MTT assay was used to check for the and cytotoxic nature of monacolin-x on Fibroblast cell lines. The cell viability was measured by MTT assay. All the cells

were treated with monacolin-x at various Concentrations (100, 200, 300, 400 and 500nM), IC₅₀ 291.4nM respectively. The % of live cells were decrease da long with increasing the concentrations of sample

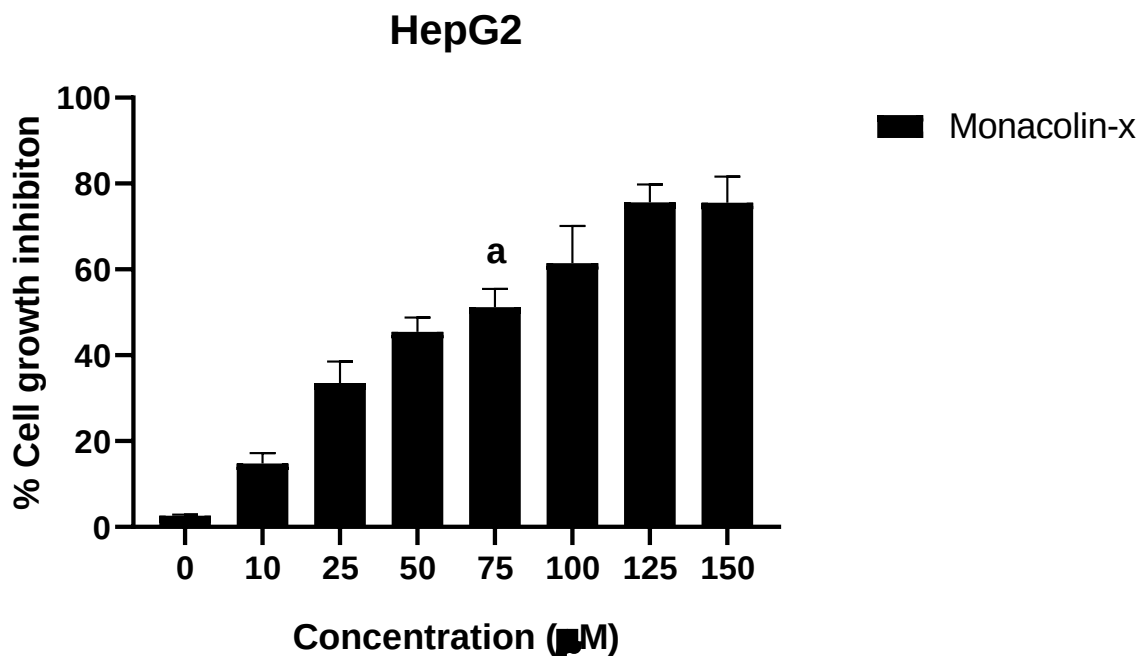


Fig:5.4 The MTT assay result of HepG2 cell line. MTT assay of monacolin-x against human liver cancer cell line at different concentrations (0-150µM). All of the measurements were done in duplicates, and results are expressed as Mean± Standard error on the mean. Statistical significance was assessed using Graph pad Prism one-way as compared to the control group. IC₅₀ value of Monacolin-x 75µM. Data are presented as the mean ± SEM values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

5.1.4 Necrosis assay

Fluorescent microscopic examination of HepG2 cells and Fibroblast cells treated with monacolin-x for 24 hours shows the formation of apoptotic bodies evidencing the apoptotic inducing role of molecule. Propidium Iodide (PI) is a nucleic acid fluorescent intercalating agent dye which is impermeable and permeable to damaged cell membrane to stain their nucleus. PI stain is used to distinguish between apoptotic cells from normal cells. PI stains the condensed nuclei of apoptotic cells. Fibroblast cells were treated with IC₅₀ concentration of monacolin-

x. (Fig.5.5-5.6) shows the morphology of HepG2 cells and fibroblast treated with monacolin-x and untreated control HepG2 cells and fibroblast cells stained with PI. The fluorescence images of the cells treated with monacolin-x showed apoptotic cell morphology, while the untreated control cells possessed intact nuclei.

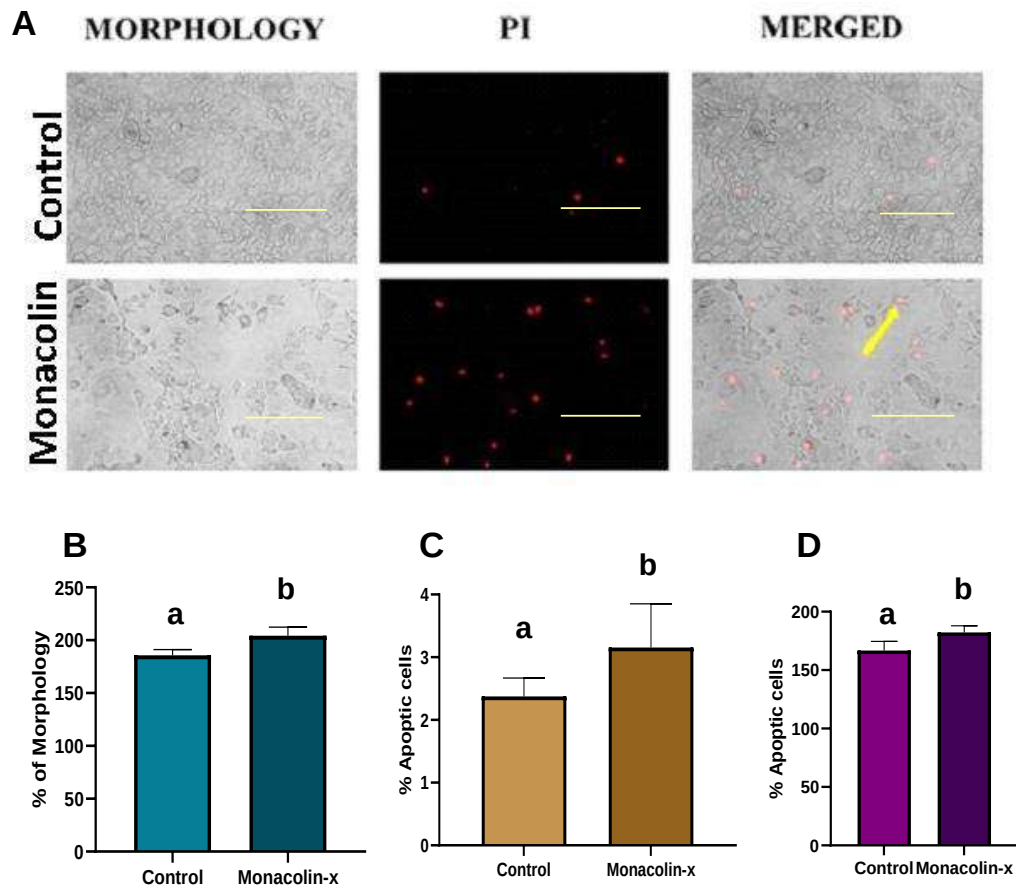


Fig:5.5 The Necrosis assay result of Fibroblast cell line. Propidium iodide staining reveals nuclear morphology of fibroblast cells exposed to dead cells on monacolin-x. (A) Control Fibroblast cells depict normal nuclei with uniform red fluorescence with minimal debris. Fibroblast cells treated with monacolin-x and observed the nuclear morphology. (B-D) The dead cells were increased along the monacolin-x treatment (Scale bar = 50 μ m). Data are presented as the mean $\pm$ SEM values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

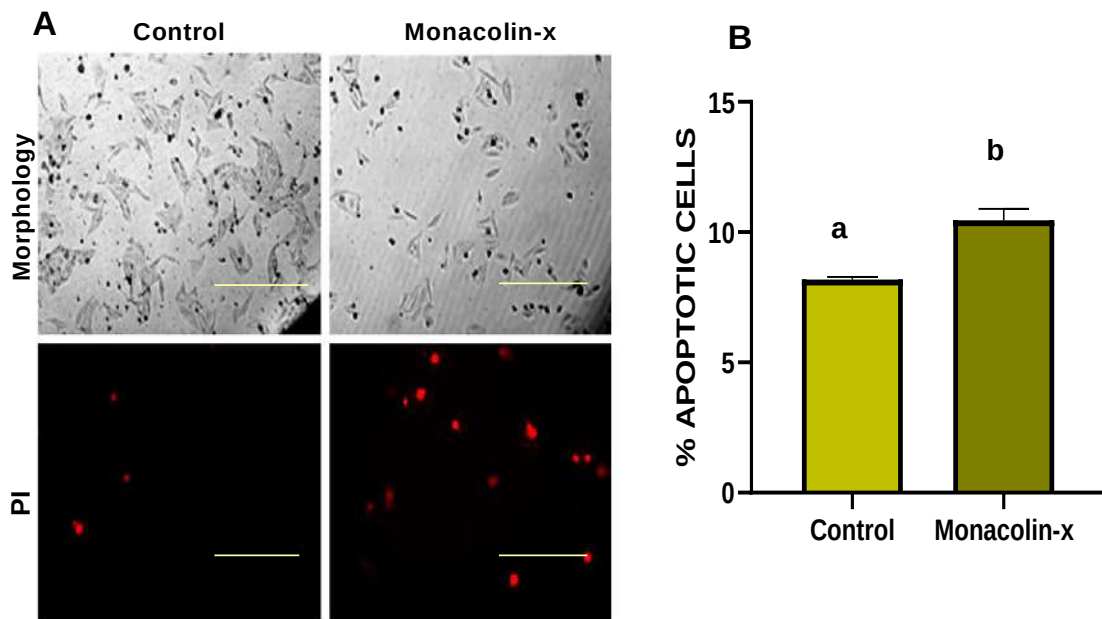


Fig:5.6 The Necrosis assay of HepG2 cell line. Propidium iodide staining reveals nuclear morphology of HepG2 cells exposed to dead cells on monacolin-x (Scale bar = 50 μ m). (A) Control HepG2 cells depict normal nuclei with uniform red fluorescence with minimal debris. HepG2 cells were treated with monacolin-x and observed the nuclear morphology of the cells. (B) The dead cells were increased along the monacolin-x treatment. The Red colour represent the cell death. Data are presented as the mean $\pm$ SEM values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

5.1.5 Apoptosis assay

Morphological changes of HepG2 cells and Fibroblast cells were observed by the treatment with monacolin-x for 24 h (Fig.5.7-5.8). After staining with AO/EB, control untreated HepG2 cells and fibroblast cells appeared green in colour with intact nuclei. After monacolin-x treatment, early apoptotic cells showed condensed nuclei and appeared light yellow in colour. Late apoptotic cells appeared in bright orange colour with condensed and fragmented nuclei. In necrotic cells, EB penetrated into the membranes of dead cells and stains their nuclei which appear red.

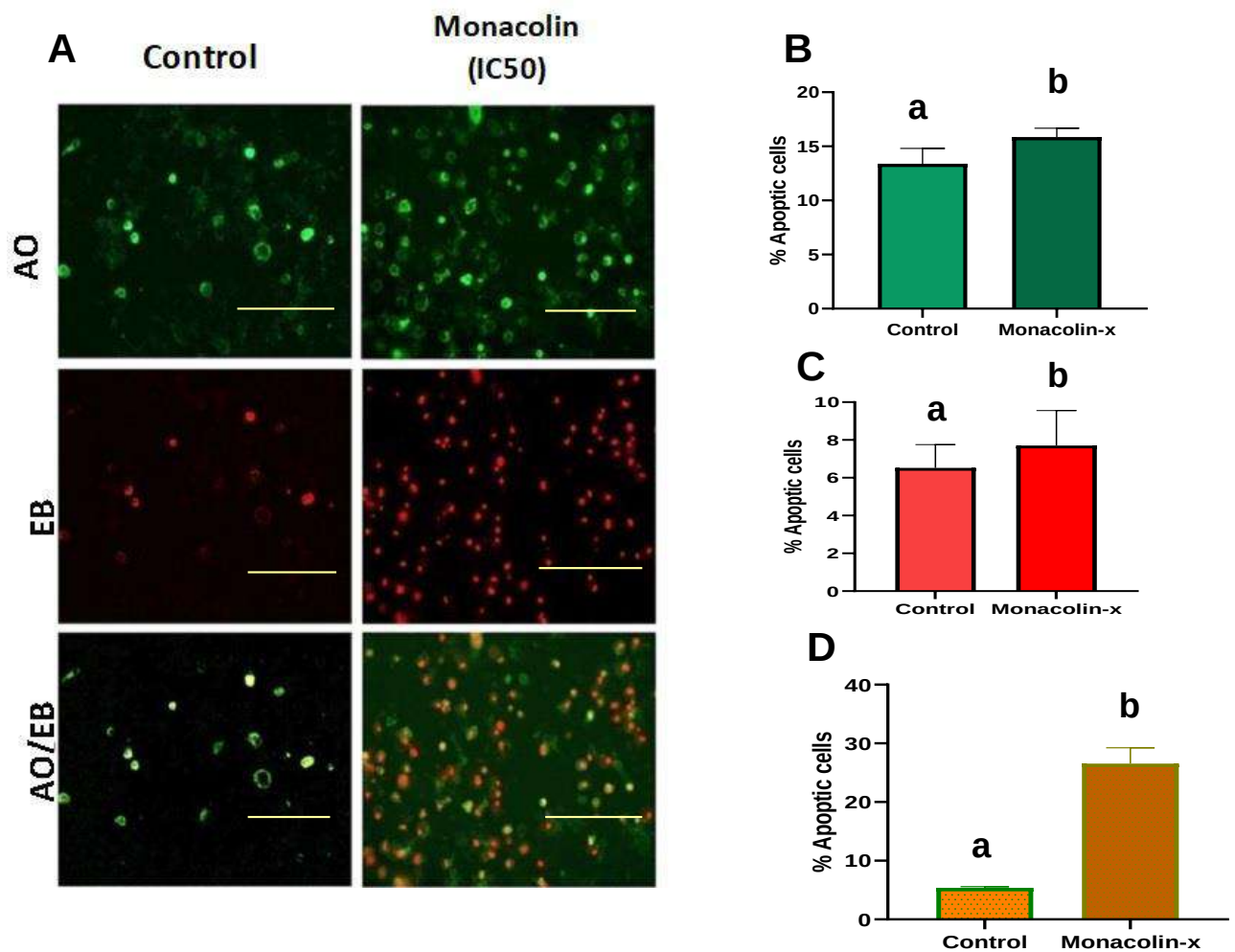


Fig:5.7 The result of apoptosis assay of Fibroblast cell line. Apoptosis is evidenced by AO/EB staining of Fibroblast cells under indicated treatment condition invitro (Scale bar = 50 μ m). The last row represents the merged images of AO and EB. (A) Control untreated fibroblast cells live cells excluded ethidium bromide and their nuclei were appeared bright green with intact structure. (B-D) represents the quantification of necrosis, early and late apoptosis cells as representations. Data are presented as the mean $\pm$ SEM values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

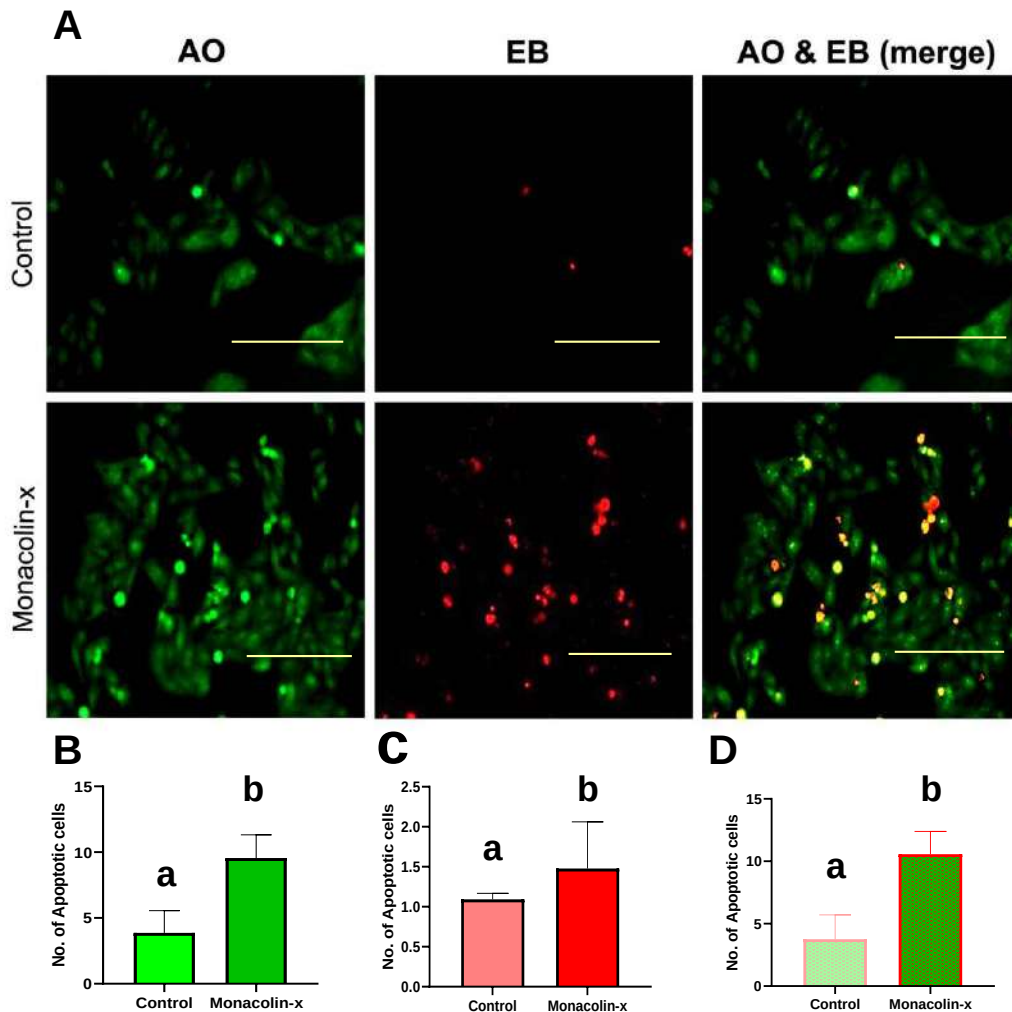


Fig:5.8 The result of apoptosis assay of HepG2 cell line. Apoptosis is evidenced by AO/ EB staining of HepG2 cells under indicated treatment condition invitro. (A) The last row represents the merged images of AO and EB. Control untreated HepG2 cells live cells excluded ethidium bromide and their nuclei were appeared bright green with intact structure (Scale bar = 50 μ m). HepG2 cells treated with monacolin-x for 24 hours. (B-D) Yellow arrow indicates early apoptotic cells (light yellow colour nuclei), Orange arrow indicates Late apoptotic cells (bright orange nuclei), whereas red colour arrow indicates necrotic cells (red coloured apoptotic bodies) at 20X magnification. Data are presented as the mean $\pm$ SEM values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

5.1.6 ROS Staining

DCFH-DA (2', 7'-dichlorodihydrofluorescein diacetate) staining is used to measure intracellular reactive oxygen species generation after 24 hours treatment with IC50 concentration of monacolin-x. DCFH-DA diffuses through the cell membrane and is hydrolyzed by an intracellular esterase to the non-fluorescent dichlorofluorescein (DCF). Then rapidly converts into the extremely fluorescent compound dichlorofluorescein in the existence of peroxidase enzyme by intracellular ROS. The fluorescent amount is directly related to the quantity of ROS produced from the cells. Fig:5.9 shows the measurement of Reactive oxygen species.

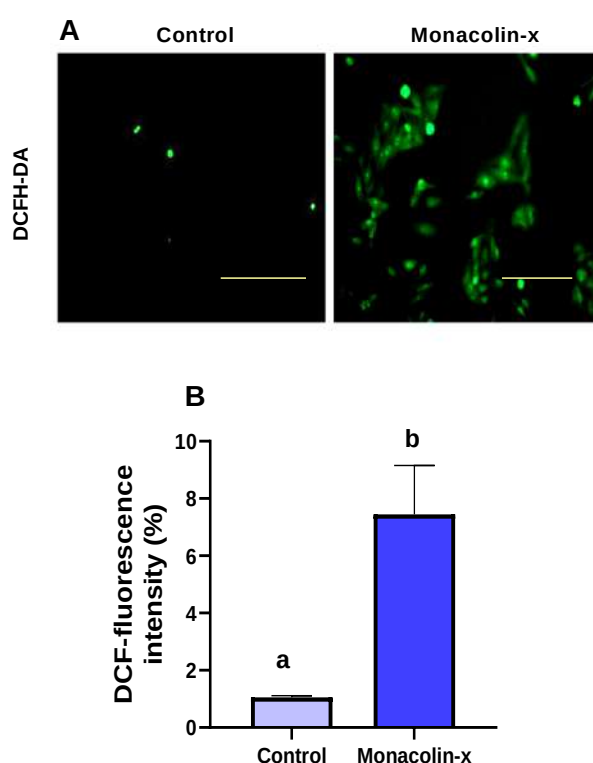


Fig:5.9 The result of DCFH-DA staining of HepG2 cell line (Scale bar = 50 μ m). Representative fluorescent images taken by a fluorescence microscope and intensity quantification by a fluorescence microplate reader for 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining in HepG2 cells after untreated control. HepG2 cells treated with monacolin-x for 24 hours (Scale bar = 50 μ m). Data are presented as the mean $\pm$ SEM values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

5.1.7 DPPH Free Radical Scavenging Assay

The DPPH radical inhibition activity of ethyl acetate extract is shown in (Fig:5.10). DPPH radical is scavenged by antioxidants through the donation of protons resulting in the reduced DPPH. The scavenging of DPPH by ethyl acetate extract was increased in a dose dependent manner. 50% of DPPH was scavenged approximately at the concentration of 24.06 µg/ml. DPPH radical inhibition of extract and standard Ascorbic acid was found almost similar.

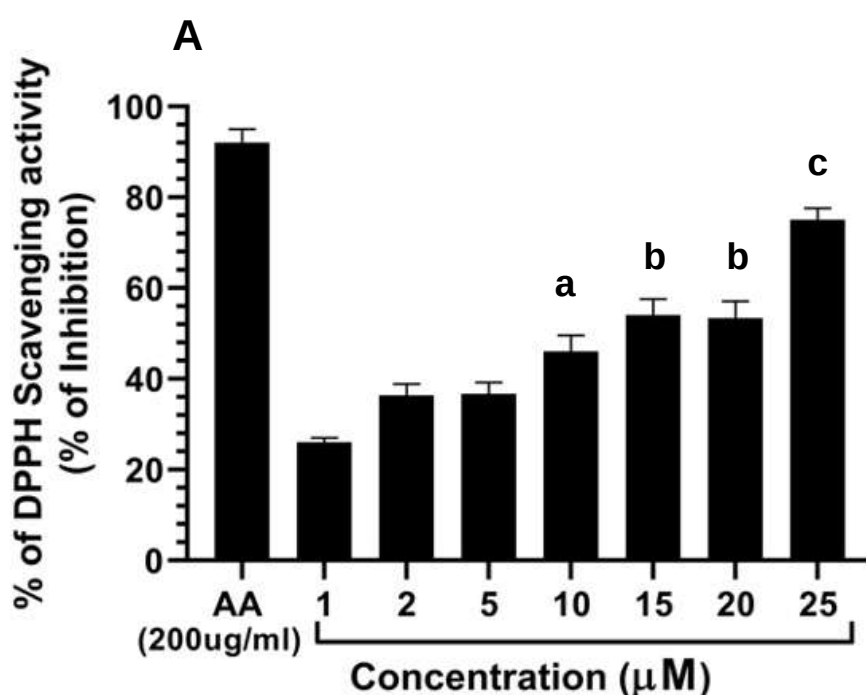


Fig:5.10 The result of the DPPH assay on HepG2 cell line. Data are presented as the mean $\pm$ SEM values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

5.1.8 Gene Expression Analysis

Gene expression were analyzed using qPCR. First, HepG2 cells were treated at IC₅₀ concentration of monacolin-x as individual and as combination then incubated for 24 hours. Then total RNA was isolated from treated cell sample using TRIzol reagent. Synthesized RNA was quantified and converted in cDNA

using cDNA synthesis kit. After cDNA synthesis, the gene expression was analyzed using the following primers for qPCR.

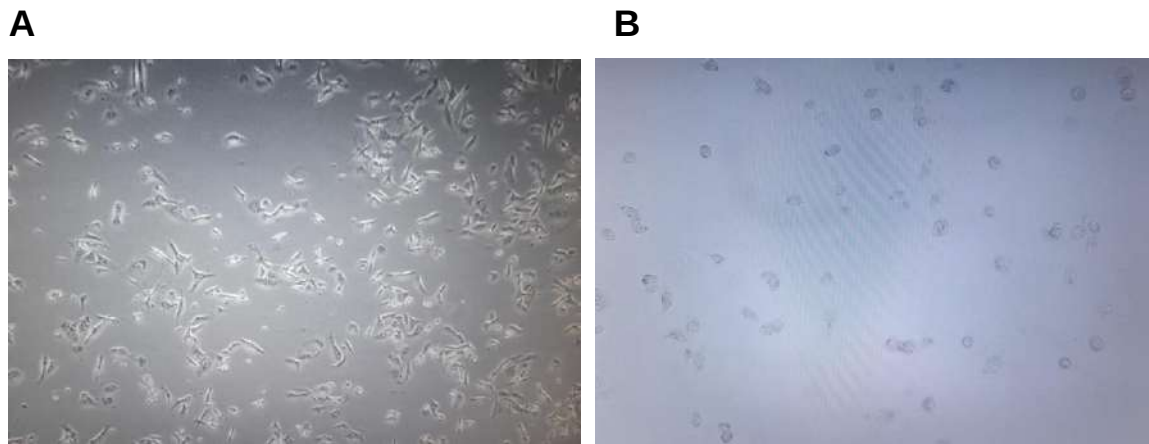


Fig:5.11 Before and After treatment of the liver cancer cells. A) Before the treatment of the cells and B) The cells treated with monacolin-x.

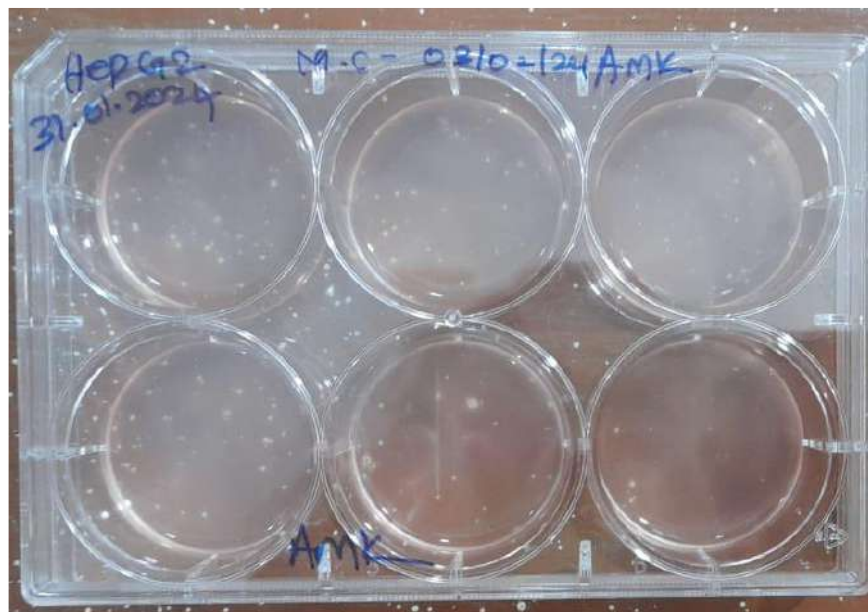


Fig:5.12 6 well plate used in the treatment for RNA isolation.



Fig:5.13 RNA isolated

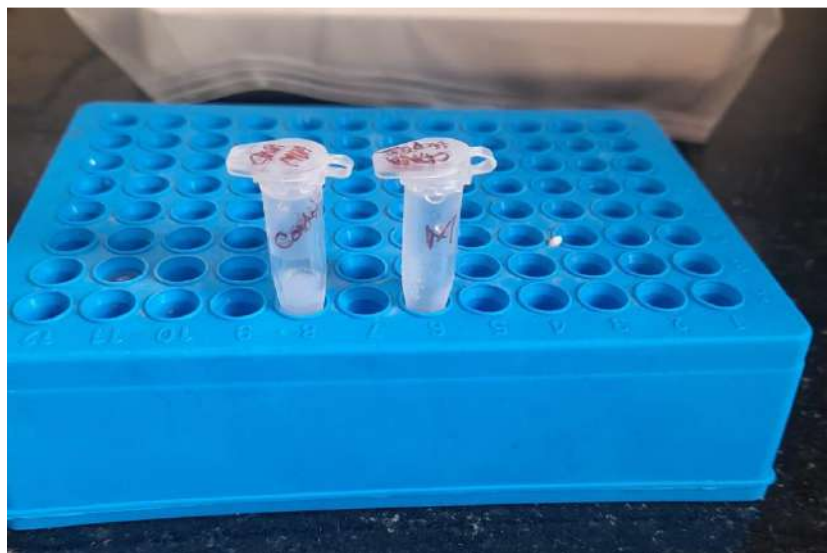


Fig:5.14 cDNA conversion.

Inflammatory gene expression by qPCR analysis

Proinflammatory cytokines like interleukin-1 beta (IL-1 beta), interleukin-6 (IL-6), and tumour necrosis factor-Beta (TNF-Beta) have a strong correlation with inflammation, according to a large body of research. Pleiotropic four-helix-bundle cytokine interleukin 6 (IL-6) performs a variety of bodily tasks. IL-6 plays a significant role in the liver in inducing the acute phase response and infection defence (References). Moreover, IL-6 is a strong hepatocyte mitogen and is essential for maintaining hepatocyte homeostasis. It is connected to both the metabolic processes of the liver and liver regeneration. On the other hand, prolonged stimulation of the IL-6 signalling pathway is harmful to the liver and may

eventually lead to the growth of liver tumours. IL-1 β (Interleukin-1beta) is a pleiotropic effect on immune cells, angiogenesis, cancer cell proliferation, migration, and metastasis. Anti-cancer treatments are able to promote IL-1 β production by cancer or immune cells, with opposite effects on cancer progression. Tumour necrosis factor Beta (TNFB) as a cytokine produced by immune cells having a capacity to suppress tumour cell proliferation and induce tumour regression

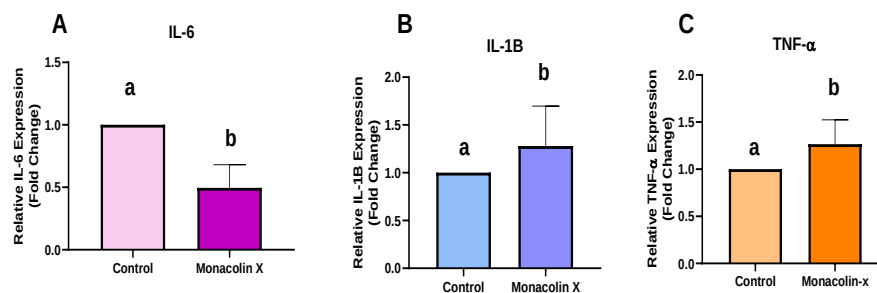


Fig:5.15 Real-time PCR Analysis for IL-6, IL-1 β and TNF- α gene expression after treatment of HepG2 cells with monacolin-x and positive standard control GAPDH for 24 hours. A) The relative gene level of IL-6 significant with control as no significance. B) The relative gene level of IL-1 β significant with control as no significance. C) The relative gene expressions level of TNF- α significant with control at ($p < 0.05$). Data are presented as the mean $\pm$ SEM values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

IL-6 (Interleukin 6) is one of the cytokine produced from inflammatory cell which promote the cancer development and inflammation. In our studies increased expression of the IL-6 in cancer cell whereas decrease the treatment. So, Monacolin x can able to treated the liver cancer cell. IL-1 β (Interleukin-1beta) is apleiotropic effects on immune cells, angiogenesis, cancer cell proliferation, migration, and metastasis. Anti-cancer treatments are able to promote IL-1 β production by cancer or immune cells, with opposite effects on cancer progression. In our studies increased expression of the IL-1 β in cancer cell whereas increase the treatment. So, Monacolin x can able to treated the liver cancer cell. Tumor necrosis factor alpha (TNF- α) as a cytokine produced by immune cells having a capacity to suppress tumor cell proliferation and induce

tumor regression. In our studies increased expression of the TNF- α in cancer cell whereas increase the treatment.

Apoptosis gene expression by qPCR analysis

qPCR analysis of Bcl2 and Bax was analyzed in HepG2 cells treated with monacolin-x and positive control GADPH. The mRNA expression level of Bcl2 and Bax significantly altered during the treatment with monacolin-x (Fig.5.11). The decreased mRNA level of anti-apoptotic gene Bcl2 and significant increase in the mRNA level of pro-apoptotic gene Bax at 24 h treatment with monacolin-x.

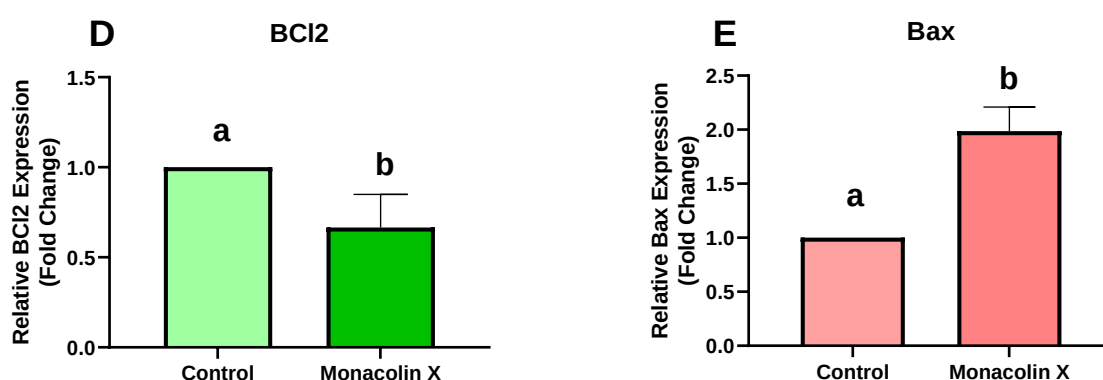


Fig:5.16 Real-time PCR Analysis for Bax, and Bcl-2, mRNA expressions after treatment of HepG2 cells with monacolin-x positive standard control GADPH for 24 hours. A) The relative mRNA level of Bcl2 significant with control as no significance. B) The relative mRNA level of Bax significant with control at ($p < 0.05$). Data are presented as the mean $\pm$ SEM values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

While Bax homodimers act as apoptosis inducers, Bcl2 heterodimer formation evokes a survival signal for the cells. Both Bcl2 and Bax are transcriptional targets for the tumour suppressor protein, p53, which induces cell cycle arrest or apoptosis in response to DNA damage. In our studies decreased expression of the Bcl-2 whereas increased the treatment and increased expression of the Bax whereas increased the treatment. This pattern of gene expression strongly supports with the protein expression of apoptosis inducing role of monacolin-x

through up-regulation of Bax while supporting the down regulation of Bcl2 expression.

5.2 *In vivo* studies

5.2.1 *Experimental design*

The experimented groups were divided into two group1 served as control zebrafish embryos. Whereas group 2 served monacolin-x treated at different concentration for different time intervals.

5.2.2 *Embryotoxicity*

The zebrafish embryo were used to establish the cytotoxic effect of monacolin-x at the early stage development. Zebrafish embryos were divided in individual wells of the 6-well plate, with each well containing the E3 embryo medium. Different concentrations of monacolin-x were added in each well, leaving some wells as controls. The zebrafish embryos were administrated the different time interval 0,24,48,72 and 96 hours. The plates were incubated at the appropriate temperature and under the proper lighting conditions for zebrafish embryos (typically Room Temperature with a 14/10-hour light/dark cycle).

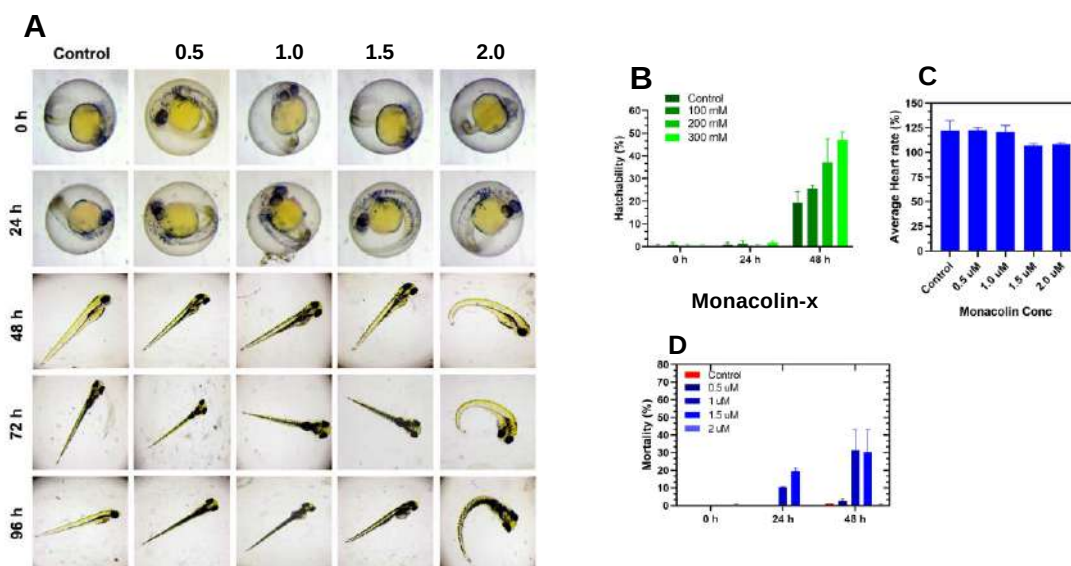


Fig:5.17 Toxic effects of monacolin-x on the development of zebrafish embryos. Each stage of the normal development process of embryos in the

control. Typical pictures of each stage of the development process of embryos at various concentrations (0.5, 1.0, 1.5 and 2.0 μ M). Heartbeat rate of the zebrafish embryos after 48hrs in different concentrations (0.5, 1.0, 1.5 and 2.0 μ M) of Monacolin-x. Percent. Hatchability and Mortality of the embryos with different concentrations of monacolin-x at various concentrations (0.5, 1.0, 1.5 and 2.0 μ M).

5.3 Statistical analysis

In this study we used for statistical analysis by using software is called Graph pad prism. Data were analyzed by one-way ANOVA, followed by Tukey's post-hoc unpaired test for comparisons among groups. Few experiments the values were compared with Student T- Test unless mentioned. Comparisons with p-values $\leq$ 0.05 were considered statistically significant.

DISCUSSION

The cytotoxic activity of monacolin-x as individual were assessed using MTT assay against HepG2 (Human liver cancer cell line) monacolin-x 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 IC₅₀ value 75µM on HepG2 cells respectively (*Niu and Wang, 2015*). monacolin-x alone also showed a decrease in cell viability on HepG2 cells. Statistical analysis suggests that monacolin-x shows significant difference in treatment on HepG2 cells. In a previous study by (*Nagabishkek and Madankumar, 2019*) reported that the IC₅₀ value of monacolin-x in MCF-7 and MDA-MB-231 was found to be 42.87 µM and 48.68 µM respectively. A study reported monacolin-x showed synergistic effect HepG2 cells was observed when Fa value was <0.5 showed additive effect (CI=1). Apoptosis induction was confirmed by PI and AO/EB staining, where the IC₅₀ value was used to evaluate the apoptosis induced by monacolin-x on HepG2 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 (*Zoi Anastasiadi et al,2016*). The percentage of apoptotic nuclei was considerably increased. AO/EB staining enables to distinguish between necrotic cells, early apoptosis cells and late apoptosis cells. monacolin-x treatment indicated higher proportion of late apoptotic cells confirmed by AO/EB staining. Thus, monacolin-x 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 monacolin-x treatment shows significantly increased levels of ROS generation (*Shai Libson et al 2014*). In this study, the antioxidative property was assessed using DPPH for measuring radical scavenging activity of monacolin-x. The antioxidant assay results revealed that the monacolin-x exhibited a linear increase in DPPH radical scavenging activity with respect to dose dependent manner (*Kolak A et al 2017*).

A diverse range of genes participate in the process of angiogenesis and the most researched pathways for angiogenesis. 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 tumour angiogenesis. Pro-inflammatory cytokines like interleukin IL-1 β and IL-6 function through different mediators, including tumour necrosis factor TNF- α , to either enhance or suppress angiogenesis, depending on the concentration and duration of exposure to cytokines. Gene expression analysis using qRT-PCR results revealed that monacolin-x treatment led to significant downregulated the mRNA expression of genes TNF- α , IL-6, IL-1 β and suggesting its role in down regulation of inflammatory and angiogenesis pathways (G Karanam, MK Arumugam, 2020).

Further, the toxicity study on zebrafish embryos by investigating the embryotoxic and teratogenic effects of the monacolin-X revealed that the 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 monacolin-x significantly inhibited ROS generation. Additionally, exposure significantly disturbed the antioxidant defense system and increased ROS in zebrafish larvae (Nagabhishek et al., 2019b).

Overall, the findings from the study provide therapeutic potential of monacolin-x 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 monacolin-x as potential therapeutic application in treatment for Human liver cancer cell (Nagabhishek and Madankumar, 2019).

CHAPTER 6

SUMMARY AND CONCLUSION

The MTT assay revealed a significant dose-dependent decrease in cell viability upon treatment with monacolin-x, indicating their potent antiproliferative activity against human liver cancer cell lines. Staining for apoptosis by AO/EB revealed that monacolin-x is able to induce apoptosis by early and late apoptosis. PI staining for necrosis or cell death and morphology change revealed that during monacolin-x treatment the cell membrane damage, cell shrinkage and morphology change was observed. General chemotherapy drugs induce ROS generation in cancer cells which kills them. We estimated ROS generation in breast cancer cells by DCHF-DA staining where the fluorescent dye binds to the ROS. PCR studies were done to show the drug upregulated pro-apoptotic gene BAX and down regulation of anti-apoptotic gene BCL2 with monacolin-x treatment of human liver cancer cells. PCR studies were done to show the drug downregulated pro-inflammatory markers like TNF- α , NF κ B, IL-1 β and apoptosis likes Bax and Bcl-2 which indicates the therapeutic potential of monacolin-x.

REFERENCES

- Afify, S.M., Seno, M., 2019. Conversion of Stem Cells to Cancer Stem Cells: Undercurrent of Cancer Initiation. *Cancers (Basel)* 11.
- Alexandrov, L.B., Ju, Y.S., Haase, K., Van Loo, P., Martincorena, I., Nik-Zainal, S., Totoki, Y., Fujimoto, A., Nakagawa, H., Shibata, T., Campbell, P.J., Vineis, P., Phillips, D.H., Stratton, M.R., 2016. Mutational signatures associated with tobacco smoking in human cancer. *Science* 354, 618-622.
- Altman, B.J., Stine, Z.E., Dang, C.V., 2016. From Krebs to clinic: glutamine metabolism to cancer therapy. *Nat Rev Cancer* 16, 619-634.
- Altorki, N., Wang, X., Kozono, D., Watt, C., Landrenau, R., Wigle, D., Port, J., Jones, D.R., Conti, M., Ashrafi, A.S., Liberman, M., Yasufuku, K., Yang, S., Mitchell, J.D., Pass, H., Keenan, R., Bauer, T., Miller, D., Kohman, L.J., Stinchcombe, T.E., Vokes, E., 2023. Lobar or Sublobar Resection for Peripheral Stage IA Non-Small-Cell Lung Cancer. *N Engl J Med* 388, 489-498.
- Amjad, M.T., Chidharla, A., Kasi, A., 2024. Cancer Chemotherapy, StatPearls, Treasure Island (FL) ineligible companies. Disclosure: Anusha Chidharla declares no relevant financial relationships with ineligible companies. Disclosure: Anup Kasi declares no relevant financial relationships with ineligible companies.
- 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.
- Anand, U., Dey, A., Chandel, A.K.S., Sanyal, R., Mishra, A., Pandey, D.K., De Falco, V., Upadhyay, A., Kandimalla, R., Chaudhary, A., Dhanjal, J.K., Dewanjee, S., Vallamkondu, J., Perez de la Lastra, J.M., 2023. Cancer chemotherapy and beyond: Current status, drug candidates, associated risks and progress in targeted therapeutics. *Genes Dis* 10, 1367-1401.
- Barbier, L., Ferhat, M., Salame, E., Robin, A., Herbelin, A., Gombert, J.M., Silvain, C., Barbarin, A., 2019. Interleukin-1 Family Cytokines: Keystones in Liver Inflammatory Diseases. *Front Immunol* 10, 2014.

- Baskar, R., Dai, J., Wenlong, N., Yeo, R., Yeoh, K.W., 2014. Biological response of cancer cells to radiation treatment. *Front Mol Biosci* 1, 24.
- Basu, A.K., 2018. DNA Damage, Mutagenesis and Cancer. *Int J Mol Sci* 19.
- Beccari, G., Cicognani, P., Bianchedi, G., Ciucci, G., 1986. [Hypocalcemia and muscular exhaustibility]. *Minerva Med* 77, 2191.
- Benedict, M., Zhang, X., 2017. Non-alcoholic fatty liver disease: An expanded review. *World J Hepatol* 9, 715-732.
- Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., Jemal, A., 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68, 394-424.
- Brown, J.S., Amend, S.R., Austin, R.H., Gatenby, R.A., Hammarlund, E.U., Pienta, K.J., 2023. Updating the Definition of Cancer. *Mol Cancer Res* 21, 1142-1147.
- Buzun, K., Bielawska, A., Bielawski, K., Gornowicz, A., 2020. DNA topoisomerases as molecular targets for anticancer drugs. *J Enzyme Inhib Med Chem* 35, 1781-1799.
- Carter, Y., Sippel, R.S., Chen, H., 2014. Hypothyroidism after a cancer diagnosis: etiology, diagnosis, complications, and management. *Oncologist* 19, 34-43.
- Chaiswing, L., St Clair, W.H., St Clair, D.K., 2018. Redox Paradox: A Novel Approach to Therapeutics-Resistant Cancer. *Antioxid Redox Signal* 29, 1237-1272.
- Chaput, G., Sumar, N., 2022. Endocrine therapies for breast and prostate cancers: Essentials for primary care. *Can Fam Physician* 68, 271-276.
- Chehelgerdi, M., Chehelgerdi, M., Allela, O.Q.B., Pecho, R.D.C., Jayasankar, N., Rao, D.P., Thamaraikani, T., Vasanthan, M., Viktor, P., Lakshmaiya, N., Saadh, M.J., Amajd, A., Abo-Zaid, M.A., Castillo-Acobo, R.Y., Ismail, A.H., Amin, A.H., Akhavan-Sigari, R., 2023. Progressing nanotechnology to improve targeted cancer treatment: overcoming hurdles in its clinical implementation. *Mol Cancer* 22, 169.

- Chidambaranathan-Reghupaty, S., Fisher, P.B., Sarkar, D., 2021. Hepatocellular carcinoma (HCC): Epidemiology, etiology and molecular classification. *Adv Cancer Res* 149, 1-61.
- Cicero, A.F.G., Fogacci, F., Banach, M., 2019. Red Yeast Rice for Hypercholesterolemia. *Methodist Debaque Cardiovasc J* 15, 192-199.
- Cui, J., Ding, R., Liu, H., Ma, M., Zuo, R., Liu, X., 2024. Trends in the incidence and survival of cancer in individuals aged 55 years and older in the United States, 1975-2019. *BMC Public Health* 24, 72.
- De Marchi, A., Pozza, S., Charrier, L., Cannone, F., Cavallo, F., Linari, A., Piana, R., Genio, I., Balocco, P., Masse, A., 2020. Small Subcutaneous Soft Tissue Tumors (<5 cm) Can Be Sarcomas and Contrast-Enhanced Ultrasound (CEUS) Is Useful to Identify Potentially Malignant Masses. *Int J Environ Res Public Health* 17.
- Debela, D.T., Muzazu, S.G., Heraro, K.D., Ndalama, M.T., Mesele, B.W., Haile, D.C., Kitui, S.K., Manyazewal, T., 2021. New approaches and procedures for cancer treatment: Current perspectives. *SAGE Open Med* 9, 20503121211034366.
- Dominiak, A., Chelstowska, B., Olejarz, W., Nowicka, G., 2020. Communication in the Cancer Microenvironment as a Target for Therapeutic Interventions. *Cancers (Basel)* 12.
- Dyke, J.G., Weaver, I.S., 2013. The emergence of environmental homeostasis in complex ecosystems. *PLoS Comput Biol* 9, e1003050.
- Elmore, S., 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 35, 495-516.
- 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.
- Feitelson, M.A., Arzumanyan, A., Kulathinal, R.J., Blain, S.W., Holcombe, R.F., Mahajna, J., Marino, M., Martinez-Chantar, M.L., Nawroth, R., Sanchez-Garcia, I., Sharma, D., Saxena, N.K., Singh, N., Vlachostergios, P.J., Guo, S., Honoki, K., Fujii, H., Georgakilas, A.G., Bilsland, A., Amedei, A.,

- Niccolai, E., Amin, A., Ashraf, S.S., Boosani, C.S., Guha, G., Ciriolo, M.R., Aquilano, K., Chen, S., Mohammed, S.I., Azmi, A.S., Bhakta, D., Halicka, D., Keith, W.N., Newsheer, S., 2015. Sustained proliferation in cancer: Mechanisms and novel therapeutic targets. *Semin Cancer Biol* 35 Suppl, S25-S54.
- Ferguson, L.R., Chen, H., Collins, A.R., Connell, M., Damia, G., Dasgupta, S., Malhotra, M., Meeker, A.K., Amedei, A., Amin, A., Ashraf, S.S., Aquilano, K., Azmi, A.S., Bhakta, D., Bilsland, A., Boosani, C.S., Chen, S., Ciriolo, M.R., Fujii, H., Guha, G., Halicka, D., Helferich, W.G., Keith, W.N., Mohammed, S.I., Niccolai, E., Yang, X., Honoki, K., Parslow, V.R., Prakash, S., Rezazadeh, S., Shackelford, R.E., Sidransky, D., Tran, P.T., Yang, E.S., Maxwell, C.A., 2015. Genomic instability in human cancer: Molecular insights and opportunities for therapeutic attack and prevention through diet and nutrition. *Semin Cancer Biol* 35 Suppl, S5-S24.
- Folkman, J., 1971. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 285, 1182-1186.
- Garcia, E.J., Lawson, D., Cotsonis, G., Cohen, C., 2002. Hepatocellular carcinoma and markers of apoptosis (bcl-2, bax, bcl-x): prognostic significance. *Appl Immunohistochem Mol Morphol* 10, 210-217.
- Garg, T., Shrigiriwar, A., Habibollahi, P., Cristescu, M., Liddell, R.P., Chapiro, J., Inglis, P., Camacho, J.C., Nezami, N., 2022. Intraarterial Therapies for the Management of Hepatocellular Carcinoma. *Cancers (Basel)* 14.
- Garner, W.B., Smith, B.D., Ludmir, E.B., Wakefield, D.V., Shabason, J., Williams, G.R., Martin, M.Y., Wang, Y., Ballo, M.T., VanderWalde, N.A., 2023. Predicting future cancer incidence by age, race, ethnicity, and sex. *Journal of Geriatric Oncology* 14.
- Gosalia, A.J., Martin, P., Jones, P.D., 2017. Advances and Future Directions in the Treatment of Hepatocellular Carcinoma. *Gastroenterol Hepatol (N Y)* 13, 398-410.

- Griscom, J.T., Wolf, P.S., 2024. Liver Metastasis, StatPearls, Treasure Island (FL) ineligible companies. Disclosure: Patrick Wolf declares no relevant financial relationships with ineligible companies.
- Gutschner, T., Diederichs, S., 2012. The hallmarks of cancer: a long non-coding RNA point of view. *RNA Biol* 9, 703-719.
- Hammarlund, E.U., Amend, S.R., Pienta, K.J., 2020. The issues with tissues: the wide range of cell fate separation enables the evolution of multicellularity and cancer. *Med Oncol* 37, 62.
- Harrop, J.P., Dean, J.A., Paskett, E.D., 2011. Cancer survivorship research: a review of the literature and summary of current NCI-designated cancer center projects. *Cancer Epidemiol Biomarkers Prev* 20, 2042-2047.
- He, Y., Sun, M.M., Zhang, G.G., Yang, J., Chen, K.S., Xu, W.W., Li, B., 2021. Targeting PI3K/Akt signal transduction for cancer therapy. *Signal Transduct Target Ther* 6, 425.
- Hillen, F., Griffioen, A.W., 2007. Tumour vascularization: sprouting angiogenesis and beyond. *Cancer Metastasis Rev* 26, 489-502.
- Hoage, T., Ding, Y., Xu, X., 2012. Quantifying cardiac functions in embryonic and adult zebrafish. *Methods Mol Biol* 843, 11-20.
- Hong, M.Y., Seeram, N.P., Zhang, Y., Heber, D., 2008. Anticancer effects of Chinese red yeast rice versus monacolin K alone on colon cancer cells. *J Nutr Biochem* 19, 448-458.
- Hora, S., Wuestefeld, T., 2023. Liver Injury and Regeneration: Current Understanding, New Approaches, and Future Perspectives. *Cells* 12.
- Jang, M., Kim, S.S., Lee, J., 2013. Cancer cell metabolism: implications for therapeutic targets. *Exp Mol Med* 45, e45.
- Jiang, J.F., Lao, Y.C., Yuan, B.H., Yin, J., Liu, X., Chen, L., Zhong, J.H., 2017. Treatment of hepatocellular carcinoma with portal vein tumor thrombus: advances and challenges. *Oncotarget* 8, 33911-33921.

- Jiang, K., Al-Diffhala, S., Centeno, B.A., 2018. Primary Liver Cancers-Part 1: Histopathology, Differential Diagnoses, and Risk Stratification. *Cancer Control* 25, 1073274817744625.
- Kalra, A., Yetiskul, E., Wehrle, C.J., Tuma, F., 2024. Physiology, Liver, StatPearls, Treasure Island (FL) ineligible companies. Disclosure: Ekrem Yetiskul declares no relevant financial relationships with ineligible companies. Disclosure: Chase Wehrle declares no relevant financial relationships with ineligible companies. Disclosure: Faiz Tuma declares no relevant financial relationships with ineligible companies.
- Karanam, G., Arumugam, M.K., 2022. Potential anticancer effects of cyclo(-Pro-Tyr) against N-diethyl nitrosamine induced hepatocellular carcinoma in mouse through PI3K/AKT signaling. *Environ Toxicol* 37, 256-269.
- Khaddour, K., Hana, C.K., Mewawalla, P., 2024. Hematopoietic Stem Cell Transplantation, StatPearls, Treasure Island (FL) ineligible companies. Disclosure: Caroline Hana declares no relevant financial relationships with ineligible companies. Disclosure: Prerna Mewawalla declares no relevant financial relationships with ineligible companies.
- Khan, Y.S., Farhana, A., 2024. Histology, Cell, StatPearls, Treasure Island (FL) ineligible companies. Disclosure: Aisha Farhana declares no relevant financial relationships with ineligible companies.
- Kim, S.K., Cho, S.W., 2022. The Evasion Mechanisms of Cancer Immunity and Drug Intervention in the Tumor Microenvironment. *Front Pharmacol* 13, 868695.
- Korde, L.A., Somerfield, M.R., Carey, L.A., Crews, J.R., Denduluri, N., Hwang, E.S., Khan, S.A., Loibl, S., Morris, E.A., Perez, A., Regan, M.M., Spears, P.A., Sudheendra, P.K., Symmans, W.F., Yung, R.L., Harvey, B.E., Hershman, D.L., 2021. Neoadjuvant Chemotherapy, Endocrine Therapy, and Targeted Therapy for Breast Cancer: ASCO Guideline. *J Clin Oncol* 39, 1485-1505.
- Krzyszczuk, P., Acevedo, A., Davidoff, E.J., Timmins, L.M., Marrero-Berrios, I., Patel, M., White, C., Lowe, C., Sherba, J.J., Hartmanshenn, C., O'Neill, K.M., Balter, M.L., Fritz, Z.R., Androulakis, I.P., Schloss, R.S., Yarmush,

- M.L., 2018. The growing role of precision and personalized medicine for cancer treatment. *Technology (Singap World Sci)* 6, 79-100.
- Kumar, A.R., Devan, A.R., Nair, B., Vinod, B.S., Nath, L.R., 2021. Harnessing the immune system against cancer: current immunotherapy approaches and therapeutic targets. *Mol Biol Rep* 48, 8075-8095.
- Kumar, R., Srivastava, R., Srivastava, S., 2015. Detection and Classification of Cancer from Microscopic Biopsy Images Using Clinically Significant and Biologically Interpretable Features. *J Med Eng* 2015, 457906.
- Leong, S.P., Naxerova, K., Keller, L., Pantel, K., Witte, M., 2022. Molecular mechanisms of cancer metastasis via the lymphatic versus the blood vessels. *Clin Exp Metastasis* 39, 159-179.
- Lewis, S., Dawson, L., Barry, A., Stanescu, T., Mohamad, I., Hosni, A., 2022. Stereotactic body radiation therapy for hepatocellular carcinoma: From infancy to ongoing maturity. *JHEP Rep* 4, 100498.
- Li, Q., Cao, M., Lei, L., Yang, F., Li, H., Yan, X., He, S., Zhang, S., Teng, Y., Xia, C., Chen, W., 2022. Burden of liver cancer: From epidemiology to prevention. *Chin J Cancer Res* 34, 554-566.
- Liberti, M.V., Locasale, J.W., 2016. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem Sci* 41, 211-218.
- Lugano, R., Ramachandran, M., Dimberg, A., 2020. Tumor angiogenesis: causes, consequences, challenges and opportunities. *Cell Mol Life Sci* 77, 1745-1770.
- Lukey, M.J., Wilson, K.F., Cerione, R.A., 2013. Therapeutic strategies impacting cancer cell glutamine metabolism. *Future Med Chem* 5, 1685-1700.
- Lurje, I., Czigany, Z., Bednarsch, J., Roderburg, C., Isfort, P., Neumann, U.P., Lurje, G., 2019. Treatment Strategies for Hepatocellular Carcinoma (-) a Multidisciplinary Approach. *Int J Mol Sci* 20.
- Mattiuzzi, C., Lippi, G., 2019. Current Cancer Epidemiology. *J Epidemiol Glob Health* 9, 217-222.

- Mercadante, A.A., Kasi, A., 2024. Genetics, Cancer Cell Cycle Phases, StatPearls, Treasure Island (FL) ineligible companies. Disclosure: Anup Kasi declares no relevant financial relationships with ineligible companies.
- Min, H.Y., Lee, H.Y., 2022. Molecular targeted therapy for anticancer treatment. *Exp Mol Med* 54, 1670-1694.
- Mirzaei, R., Sabokroo, N., Ahmadyousefi, Y., Motamedi, H., Karampoor, S., 2022. Immunometabolism in biofilm infection: lessons from cancer. *Mol Med* 28, 10.
- Moloudi, K., Abrahamse, H., George, B.P., 2023. Photodynamic therapy induced cell cycle arrest and cancer cell synchronization: review. *Front Oncol* 13, 1225694.
- Mukhtar, E., Adhami, V.M., Mukhtar, H., 2014. Targeting microtubules by natural agents for cancer therapy. *Mol Cancer Ther* 13, 275-284.
- Mun, K., Han, J., Roh, P., Park, J., Kim, G., Hur, W., Jang, J., Choi, J., Yoon, S., You, Y., Choi, H., Sung, P., 2023. Isolation and characterization of cancer-associated fibroblasts in the tumor microenvironment of hepatocellular carcinoma. *J Liver Cancer* 23, 341-349.
- Myojin, Y., Kodama, T., Sakamori, R., Maesaka, K., Matsumae, T., Sawai, Y., Imai, Y., Ohkawa, K., Miyazaki, M., Tanaka, S., Mita, E., Tawara, S., Yakushijin, T., Nozaki, Y., Hagiwara, H., Tahata, Y., Yamada, R., Hikita, H., Tatsumi, T., Takehara, T., 2022. Interleukin-6 Is a Circulating Prognostic Biomarker for Hepatocellular Carcinoma Patients Treated with Combined Immunotherapy. *Cancers (Basel)* 14.
- Nagabhishek, S.N., Madan Kumar, A., B, S., Balakrishnan, A., Katakia, Y.T., Chatterjee, S., Nagasundaram, N., 2019a. A marine sponge associated fungal metabolite monacolin X suppresses angiogenesis by down regulating VEGFR2 signaling. *RSC Adv* 9, 26646-26667.
- Nagabhishek, S.N., Madan Kumar, A., B, S., Balakrishnan, A., Katakia, Y.T., Chatterjee, S., Nagasundaram, N., 2019b. A marine sponge associated fungal metabolite monacolin X suppresses angiogenesis by down regulating VEGFR2 signaling. *RSC Advances* 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.
- Ni, J., Zhang, L., 2020. Cancer Cachexia: Definition, Staging, and Emerging Treatments. *Cancer Manag Res* 12, 5597-5605.
- Nishida, N., Yano, H., Nishida, T., Kamura, T., Kojiro, M., 2006. Angiogenesis in cancer. *Vasc Health Risk Manag* 2, 213-219.
- Niu, N., Wang, L., 2015. In vitro human cell line models to predict clinical response to anticancer drugs. *Pharmacogenomics* 16, 273-285.
- Ozaki, T., Nakagawara, A., 2011. Role of p53 in Cell Death and Human Cancers. *Cancers (Basel)* 3, 994-1013.
- Pan, S., Fan, M., Liu, Z., Li, X., Wang, H., 2021. Serine, glycine and one-carbon metabolism in cancer (Review). *Int J Oncol* 58, 158-170.
- Pasquier, J., Guerrouahen, B.S., Al Thawadi, H., Ghiabi, P., Maleki, M., Abu-Kaoud, N., Jacob, A., Mirshahi, M., Galas, L., Rafii, S., Le Foll, F., Rafii, A., 2013. Preferential transfer of mitochondria from endothelial to cancer cells through tunneling nanotubes modulates chemoresistance. *J Transl Med* 11, 94.
- Pfeffer, C.M., Singh, A.T.K., 2018. Apoptosis: A Target for Anticancer Therapy. *Int J Mol Sci* 19.
- Pham-Huy, L.A., He, H., Pham-Huy, C., 2008. Free radicals, antioxidants in disease and health. *Int J Biomed Sci* 4, 89-96.
- Pinho, S.T.R., Freedman, H.I., Nani, F., 2002. A chemotherapy model for the treatment of cancer with metastasis. *Mathematical and Computer Modelling* 36, 773-803.
- Pinter, M., Trauner, M., Peck-Radosavljevic, M., Sieghart, W., 2016. Cancer and liver cirrhosis: implications on prognosis and management. *ESMO Open* 1, e000042.

- Pistritto, G., Trisciuglio, D., Ceci, C., Garufi, A., D'Orazi, G., 2016. Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. *Aging (Albany NY)* 8, 603-619.
- Pulumati, A., Pulumati, A., Dwarakanath, B.S., Verma, A., Papineni, R.V.L., 2023. Technological advancements in cancer diagnostics: Improvements and limitations. *Cancer Rep (Hoboken)* 6, e1764.
- Rao, K.V., Faso, A., 2012. Chemotherapy-induced nausea and vomiting: optimizing prevention and management. *Am Health Drug Benefits* 5, 232-240.
- Rebe, C., Ghiringhelli, F., 2020. Interleukin-1beta and Cancer. *Cancers (Basel)* 12.
- Rui, L., 2014. Energy metabolism in the liver. *Compr Physiol* 4, 177-197.
- Russo, F.P., Zanetto, A., Pinto, E., Battistella, S., Penzo, B., Burra, P., Farinati, F., 2022. Hepatocellular Carcinoma in Chronic Viral Hepatitis: Where Do We Stand? *Int J Mol Sci* 23.
- Saman, H., Raza, S.S., Uddin, S., Rasul, K., 2020. Inducing Angiogenesis, a Key Step in Cancer Vascularization, and Treatment Approaches. *Cancers (Basel)* 12.
- Sarkar, S., Horn, G., Moulton, K., Oza, A., Byler, S., Kokolus, S., Longacre, M., 2013. Cancer development, progression, and therapy: an epigenetic overview. *Int J Mol Sci* 14, 21087-21113.
- Sathishkumar, K., Chaturvedi, M., Das, P., Stephen, S., Mathur, P., 2022. Cancer incidence estimates for 2022 & projection for 2025: Result from National Cancer Registry Programme, India. *Indian J Med Res* 156, 598-607.
- Schiliro, C., Firestein, B.L., 2021. Mechanisms of Metabolic Reprogramming in Cancer Cells Supporting Enhanced Growth and Proliferation. *Cells* 10.
- Schmidt, D.R., Patel, R., Kirsch, D.G., Lewis, C.A., Vander Heiden, M.G., Locasale, J.W., 2021. Metabolomics in cancer research and emerging applications in clinical oncology. *CA Cancer J Clin* 71, 333-358.
- Seyfried, T.N., Huysentruyt, L.C., 2013. On the origin of cancer metastasis. *Crit Rev Oncog* 18, 43-73.

- Shakiba, E., Ramezani, M., Sadeghi, M., 2018. Evaluation of serum interleukin-6 levels in hepatocellular carcinoma patients: a systematic review and meta-analysis. *Clin Exp Hepatol* 4, 182-190.
- Shiravand, Y., Khodadadi, F., Kashani, S.M.A., Hosseini-Fard, S.R., Hosseini, S., Sadeghirad, H., Ladwa, R., O'Byrne, K., Kulasinghe, A., 2022. Immune Checkpoint Inhibitors in Cancer Therapy. *Curr Oncol* 29, 3044-3060.
- Shuvalov, O., Petukhov, A., Daks, A., Fedorova, O., Vasileva, E., Barlev, N.A., 2017. One-carbon metabolism and nucleotide biosynthesis as attractive targets for anticancer therapy. *Oncotarget* 8, 23955-23977.
- Siegel, R.L., Miller, K.D., Wagle, N.S., Jemal, A., 2023. Cancer statistics, 2023. *CA Cancer J Clin* 73, 17-48.
- Singh, N., Baby, D., Rajguru, J.P., Patil, P.B., Thakkannavar, S.S., Pujari, V.B., 2019. Inflammation and cancer. *Ann Afr Med* 18, 121-126.
- Stenberg, U., Ruland, C.M., Miaskowski, C., 2010. Review of the literature on the effects of caring for a patient with cancer. *Psychooncology* 19, 1013-1025.
- Stincone, A., Prigione, A., Cramer, T., Wamelink, M.M., Campbell, K., Cheung, E., Olin-Sandoval, V., Gruning, N.M., Kruger, A., Tauqeer Alam, M., Keller, M.A., Breitenbach, M., Brindle, K.M., Rabinowitz, J.D., Ralser, M., 2015. The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. *Biol Rev Camb Philos Soc* 90, 927-963.
- Sun, Y., Liu, Y., Ma, X., Hu, H., 2021. The Influence of Cell Cycle Regulation on Chemotherapy. *Int J Mol Sci* 22.
- Suresh, D., Srinivas, A.N., Kumar, D.P., 2020. Etiology of Hepatocellular Carcinoma: Special Focus on Fatty Liver Disease. *Front Oncol* 10, 601710.
- Tang, S.K.Y., Marshall, W.F., 2017. Self-repairing cells: How single cells heal membrane ruptures and restore lost structures. *Science* 356, 1022-1025.
- Torgovnick, A., Schumacher, B., 2015. DNA repair mechanisms in cancer development and therapy. *Front Genet* 6, 157.

- Torralba, D., Baixauli, F., Sanchez-Madrid, F., 2016. Mitochondria Know No Boundaries: Mechanisms and Functions of Intercellular Mitochondrial Transfer. *Front Cell Dev Biol* 4, 107.
- Valenti, D., Vacca, R.A., Moro, L., Atlante, A., 2021. Mitochondria Can Cross Cell Boundaries: An Overview of the Biological Relevance, Pathophysiological Implications and Therapeutic Perspectives of Intercellular Mitochondrial Transfer. *Int J Mol Sci* 22.
- van Zijl, F., Krupitza, G., Mikulits, W., 2011. Initial steps of metastasis: cell invasion and endothelial transmigration. *Mutat Res* 728, 23-34.
- Vanauberg, D., Schulz, C., Lefebvre, T., 2023. Involvement of the pro-oncogenic enzyme fatty acid synthase in the hallmarks of cancer: a promising target in anti-cancer therapies. *Oncogenesis* 12, 16.
- Vanneman, M., Dranoff, G., 2012. Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer* 12, 237-251.
- Wang, J., Yu, X., Peng, H., Lu, Y., Li, S., Shi, Q., Liu, J., Dong, H., Katanaev, V., Jia, L., 2021. Embedding similarities between embryos and circulating tumor cells: fundamentals of abortifacients used for cancer metastasis chemoprevention. *J Exp Clin Cancer Res* 40, 300.
- Wang, R.C., Wang, Z., 2023. Precision Medicine: Disease Subtyping and Tailored Treatment. *Cancers (Basel)* 15.
- Wang, T.H., Lin, T.F., 2007. Monascus rice products. *Adv Food Nutr Res* 53, 123-159.
- Weber, G.F., 2014. Molecular Inhibitors of Growth Signals. *Molecular Therapies of Cancer*, 163-242.
- Wondrak, G.T., 2009. Redox-directed cancer therapeutics: molecular mechanisms and opportunities. *Antioxid Redox Signal* 11, 3013-3069.
- Wu, H., Wang, M.-D., Zhu, J.-Q., Li, Z.-L., Wang, W.-Y., Gu, L.-H., Shen, F., Yang, T., 2022. Mesoporous Nanoparticles for Diagnosis and Treatment of Liver Cancer in the Era of Precise Medicine. *Pharmaceutics* 14, 1760.

- Xie, X., Li, Y., Lian, S., Lu, Y., Jia, L., 2022. Cancer metastasis chemoprevention prevents circulating tumour cells from germination. *Signal Transduct Target Ther* 7, 341.
- Yamakado, K., Hirota, S., 2015. Sub-classification of intermediate-stage (Barcelona Clinic Liver Cancer stage-B) hepatocellular carcinomas. *World J Gastroenterol* 21, 10604-10608.
- Yang, F., Teves, S.S., Kemp, C.J., Henikoff, S., 2014. Doxorubicin, DNA torsion, and chromatin dynamics. *Biochim Biophys Acta* 1845, 84-89.
- Yang, Y.M., Seki, E., 2015. TNF α in liver fibrosis. *Curr Pathobiol Rep* 3, 253-261.
- Yeo, H.Y., Liew, A.C., Chan, S.J., Anwar, M., Han, C.H., Marra, C.A., 2023. Understanding Patient Preferences Regarding the Important Determinants of Breast Cancer Treatment: A Narrative Scoping Review. *Patient Prefer Adherence* 17, 2679-2706.
- Zaefarian, F., Abdollahi, M.R., Cowieson, A., Ravindran, V., 2019. Avian Liver: The Forgotten Organ. *Animals (Basel)* 9.
- Zhang, C., Yang, M., Ericsson, A.C., 2020. The Potential Gut Microbiota-Mediated Treatment Options for Liver Cancer. *Front Oncol* 10, 524205.