

PROTECTIVE EFFECT OF MONACOLIN X AGAINST CHRONIC ETHANOL INDUCED HEPATIC STEATOSIS AND INJURY

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

By

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ms. SANGAMITHRA S (42101012)** who carried out Project entitled "**PROTECTIVE EFFECT OF MONACOLIN X AGAINST CHRONIC ETHANOL INDUCED HEPATIC STEATOSIS AND INJURY**" under the supervision of **Dr. A. Madan Kumar, Associate Professor (Research)** from September 2023 to April 2024.

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DECLARATION

I, **Ms. SANGAMITHRA S (42101012)** hereby declare that the Project Report entitled "**PROTECTIVE EFFECT OF MONACOLIN X AGAINST CHRONIC ETHANOL INDUCED HEPATIC STEATOSIS AND INJURY**" 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

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ABSTRACT

Hepatic steatosis, is one of the liver diseases, is a condition characterized by the accumulation of excess fat within liver cells, which can lead to liver enlargement and potential impairment of liver function. It is often associated with metabolic disorders such as obesity, diabetes, high cholesterol levels, and insulin resistance. On the other hand, ethanol-induced hepatic steatosis and injury occur due to chronic and excessive alcohol consumption. Ethanol is metabolized in the liver, producing toxic by-products that can damage liver cells and disrupt normal liver function. This condition is a significant health concern globally, contributing to a range of liver-related complications such as alcoholic hepatitis, fibrosis, cirrhosis, and even hepatocellular carcinoma. In the quest for effective treatments for hepatic steatosis and injury, researchers have turned their attention to Monacolin X, a natural compound with potential pharmacological properties. Monacolin X has garnered interest due to its ability to modulate angiogenesis, inhibit HMG-CoA reductase (similar to statins), and reduce oxidative stress. These mechanisms make it a promising candidate for addressing liver diseases, particularly those associated with chronic alcohol consumption. Experimental studies using zebrafish models as a platform for investigation have provided valuable insights into the protective effects of Monacolin X against ethanol-induced liver damage. Assays measuring hepatic function, oxidative stress markers, and inflammatory gene expression have shown promising results. Monacolin X treatment led to reductions in total protein levels, SGOT, and SGPT enzymes, which are indicators of liver injury. Additionally, the compound demonstrated modulation of key oxidative stress markers such as Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxide (GPx), Glutathione (GSH), and Glutathione S Transferase (GST), highlighting its potential to mitigate oxidative stress-induced liver damage. Furthermore, PCR analysis targeting inflammatory genes including NFkB, TNF-alpha, IL-6, and IL-1 beta showed a downregulation in their expression levels after Monacolin X treatment, indicating its anti-inflammatory properties. Monacolin X holds promise as a novel treatment approach that could help alleviate the burden of liver diseases associated with alcohol abuse, paving the way for improved patient outcomes and management strategies in this challenging medical domain.

Keywords: Liver Disease, ALD, Hepatic Steatosis, Monacolin X, Zebrafish.

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LIST OF ABBREVIATIONS

ADH	Alcohol Dehydrogenase
AIH	Autoimmune Hepatitis
ALD	Alcoholic Liver Disease
ALT	Alanine Amino Transferase
AMPK	AMP-activated protein kinase
ARLD	Alcohol-related Liver Disease
AST	Aspartate Amino Transferase
BCAA	Branched-chain amino acid
BSA	Bovine Serum Albumin
CAT	Catalase
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CDNB	Chloro Dinitro Benzene
CNS	Central Nervous System
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
DNA	Deoxy Ribo Nucleic Acid
DNPH	Dinitrophenyl Hydrazine
DTNB	Dithio nitrobenzoic acid
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium Bromide
EtOH	Ethanol
GABA	Gamma-aminobutyric acid
GPx	Glutathione Peroxide
GSH	Glutathione

GSSG	Glutathione disulfide
GST	Glutathione S-Transferase
H&E	Haematoxylin and Eosin Stain
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HEV	Hepatitis E virus
HSCs	Hepatic stellate cells
IAEC	Institutional Animal Ethics Committee
IL-1 β	Interleukin-1 beta
IL-6	Interleukin 6
IVC	Inferior vena cava
KCs	Kupffer cells
MMP	Mitochondrial Membrane Potential
MSCs	Mesenchymal stem cells
NAFLD	Non-alcoholic Fatty Liver Disease
NaOH	Sodium Hydroxide
NASH	Non-alcoholic steatohepatitis
NF κ B	Nuclear Factor kappa B
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NMDA	N-methyl-D-aspartate
NMR	Nuclear Magnetic Resonance spectroscopy
PBC	Primary Biliary Cholangitis
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived growth factor
PPAR α	Peroxisome proliferator-activated receptor alpha

PSC	Primary Sclerosing Cholangitis
qRT-PCR	Quantitative Real-Time-Polymerase Chain Reaction
RNA	Ribose Nucleic Acid
ROS	Reactive Oxygen Species
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulfate
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvate Transaminase
SOD	Superoxide Dismutase
SREBP-1c	Sterol regulatory element-binding protein 1c
TBE buffer	Tris-Borate-EDTA
TCA	Trichloroacetic acid
TGF- β	Transforming growth factor-beta
TLRs	Toll-line receptors
TNF- α	Tumour Necrosis Factor alpha
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization

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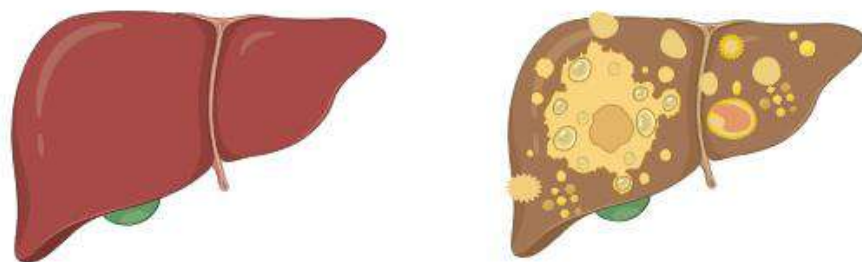
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CHAPTER 1

INTRODUCTION

1.1 LIVER DISEASE

Liver is a vital organ with impressive regenerative potential and can cure significant damage following removal. Some illnesses can over stimulate its capacity for self-healing, leading to an excessive accumulation of collagen and cellular matrix (Neshat, S.Y. *et al.*, 2021). Liver disease encompasses a wide range of conditions that affect the liver, a vital organ responsible for numerous crucial functions in the body (Byass, P. 2014). The metabolic processes to detoxification and bile production, the liver plays a central role in maintaining overall health and it also helps in storage of vitamins and minerals. Liver disease refers to any condition that affects the liver and impairs its function. Although the causes of these disorders vary, they can all harm and affect the function of liver. Liver disease can result from various causes, including viral infections, alcohol abuse, autoimmune disorders, genetic factors, obesity and exposure to toxins (Diehl, A.M. 2002). Due to the lack of available liver donors and our limited knowledge of how liver illnesses progress, liver diseases pose substantial health care costs that are still not effectively managed. Chronic illnesses that produce significant harm to liver over time.



Healthy liver

Fatty liver

Fig:1.1 : Liver disease

Early on, liver disease don't show any overt signs and grow silently. However, with lifestyle modifications, the condition can be mostly avoided, when symptoms

develop, it is likely to be due to scarring of liver from damage. The symptoms include early and later stages of liver diseases. The early symptoms were feeling generally ill and exhausted all the time, weight loss and muscular atrophy, loss of appetite, feeling nauseous and vomiting, pain in the liver area, spider-like small blood capillaries on the skin above waist level, red palms, disturbed sleep patterns. The later symptoms were severe irritation of the skin, jaundice (yellowing of the skin and whites of the eyes), and white nails. Finger tips thicken or widen (clubbed fingers), hair thinning, oedema, or swelling in the legs, ankles, and feet, abdominal enlargement (ascites), dark pee, light-coloured feces, or extremely dark, tarry stools, bleeding gums and nosebleeds frequently, easy bruising and hard to stop minor bleeding, vomit blood, cramping in the muscles often, Men with right shoulder pain may also have swollen breasts and shrinking testicles, irregular or nonexistent menstrual cycles, sexual desire loss and impotence, acute exhaustion and light-headedness (anaemia), breathlessness and an extremely fast heartbeat (tachycardia), fevers with chills and a high body temperature, disorientation, drowsiness, forgetfulness, and memory loss. Little alteration in personality, shaky hands, writing becomes hard, stumbling pace when walking, a predisposition to fall, heightened sensitivity to medications, both medicinal and recreational, heightened sensitivity to alcohol.

1.2 TYPES OF LIVER DISEASE

Acute and chronic liver diseases are two broad categories that include a variety of liver related diseases, each with its own set of characteristics and implications.

- Acute Liver Disease
- Chronic Liver Disease

1.2.1 ACUTE LIVER DISEASE

Acute liver disease is defined as the sudden onset of liver dysfunction or damage over a short period of time (Gines, P. *et al.*, 2010). Acute liver disease can be caused by an array of reasons, including viral infections (such as hepatitis A, B, and E), drugs toxicity (for example, acetaminophen overdose), autoimmune hepatitis, ischemic liver injury, and acute fatty liver during pregnancy, among others. Acute liver disease symptoms include jaundice (yellowing of the skin and eyes), stomach discomfort and swelling, nausea, vomiting, exhaustion, dark urine,

and clay-colored feces. (Schaefer, T. J., & John, S. 2023, Shah, Niraj J. *et al.*, 2023).

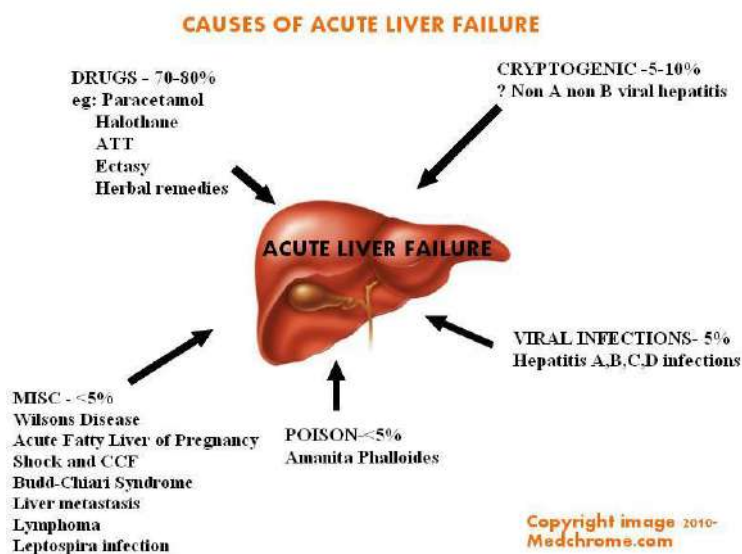


Fig:1.2 : Causes of Acute Liver Failure (Gines, P. *et al.*, 2010)

1.2.2 CHRONIC LIVER DISEASE

Chronic liver disease is characterized by long-term liver damage or dysfunction that persists for more than six months. It often progresses slowly over time and can lead to cirrhosis (scarring of the liver) and its complications (Sharma, A., & Nagalli, S. 2023). Chronic liver disease can be caused by a wide range of factors, including viral hepatitis (hepatitis B and C), alcoholism, non-alcoholic fatty liver disease (NAFLD), autoimmune hepatitis, genetic disorders (such as hemochromatosis or Wilson's disease), and certain medications or toxins (Sharma, A., & Nagalli, S. 2023). Symptoms of chronic liver disease may include fatigue, weakness, loss of appetite, weight loss, jaundice, easy bruising or bleeding, swelling in the abdomen or legs (ascites and edema), and mental confusion (hepatic encephalopathy).

There are numerous kinds of liver diseases. Some are inherited, while others are caused by viruses, other disorders, or poisons like drugs or alcohol (Sharma, A., & Nagalli, S. 2023). Liver diseases are a broad category of ailments affecting the liver, ranging from very moderate and reversible to severe and life-threatening. The other common liver diseases includes Hepatitis, Liver Cirrhosis, Alcoholic liver

disease, Non Alcoholic fatty liver disease, Auto immune liver disease and Liver cancer (Neshat, S.Y. *et al.*, 2021).

1.2.3 HEPATITIS

Hepatitis refers to inflammation of the liver and can be caused by viruses (hepatitis A, B, C, D, and E), alcohol misuse, autoimmune disorders, medications, and toxins. Hepatitis A and E are typically acute and caused by ingestion of contaminated food or water. Hepatitis B, C, and D can become chronic and may lead to liver cirrhosis or liver cancer if left untreated. WORLD HEALTH ORGANIZATION (2017). There are five sorts of hepatitis, including

HEPATITIS A

Hepatitis A is commonly transmitted by contact with contaminated food or water. Symptoms may improve without treatment, but recovery can take several weeks (Iorio, N., & John, S. 2023).

HEPATITIS B

This kind of viral hepatitis may be acute (short-term) or chronic (long-term). It is transmitted through body fluids such as blood and sperm. Hepatitis B can be treated, but it cannot be cured. Early treatment is critical for avoiding issues, so get regular checkups if you're at risk (Johnson, K.D. *et al.*, 2019).

HEPATITIS C

Hepatitis C can be either acute or chronic. Contact with hepatitis C-infected blood is a common route of transmission. While it rarely causes symptoms in the early stages, it can eventually cause chronic liver damage (Hyland, C. *et al.*, 2003, Seeff, L.B. & Hoofnagle, J.H. 2002). Hepatitis C virus infection can be confirmed with antibody to hepatitis C virus (anti-HCV). These assays can detect antibodies within 4 to 10 weeks of infection and are pretty sensitive and specific. Anti-HCV can remain negative for a few months after an acute hepatitis C virus infection, but once it appears in the blood, it remains present for life. However, it is important to remember that anti-HCV is not a protective antibody, unlike anti-HBs, and does not offer protection from subsequent exposure to the hepatitis C virus (Bajpai, M. *et al.*, 2014).

HEPATITIS D

This is a hazardous kind of hepatitis that only occurs in people with hepatitis B; it cannot be obtained on its own. It might be acute or persistent (Tripathi, N., & Mousa, O. Y. 2023).

HEPATITIS E

Hepatitis E is typically caused by ingesting polluted water. In most cases, it resolves itself within a few weeks with no long-term problems (Kamar, N. *et al.*, 2014). Hepatitis E is inflammation of the liver caused by the hepatitis E virus (HEV). The virus has at least 4 different types: genotypes 1, 2, 3 and 4. Genotypes 1 and 2 have been found only in humans. Genotypes 3 and 4 circulate in several animals including pigs, wild boars and deer without causing any disease, and occasionally infect humans.

The virus is shed in the stools of infected persons and enters the human body through the intestine. It is transmitted mainly through contaminated drinking water. The infection is usually self-limiting and resolves within 2–6 weeks. Occasionally a serious disease known as fulminant hepatitis (acute liver failure) develops, which can be fatal (Kamani, L. *et al.*, 2021).

1.2.4 FATTY LIVER DISEASE

Excess fat storage in the liver can leads fatty liver disease. Alcoholic fatty liver disease (ALD) and Non alcoholic fatty liver disease (NAFLD) comes under fatty liver diseases. Alcoholic fatty liver disease is caused by excessive alcohol consumption, while nonalcoholic fatty liver disease is caused by other factors. ALD encompasses a spectrum of liver diseases caused by excessive alcohol consumption, including fatty liver, alcoholic hepatitis, fibrosis, and cirrhosis.

The severity of ALD depends on the amount and duration of alcohol consumption (Lucey, M.R. *et al.*, 2009). NAFLD is characterized by excessive fat accumulation in the liver, not due to excessive alcohol consumption.

Non-alcoholic steatohepatitis (NASH) is a more severe form of NAFLD characterized by inflammation and liver cell damage, which can progress to cirrhosis and liver cancer. Both NAFLD and NASH are closely associated with obesity, insulin resistance, and metabolic syndrome (Younossi, Z.M., *et al.* 2016).

1.2.5 AUTOIMMUNE DISEASES

Autoimmune liver diseases include autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). These conditions occur when the body's immune system attacks the liver cells, bile ducts, or both (Manns, M.P. *et al.*, 2010)

1.2.6 LIVER CANCER (HEPATOCELLULAR CARCINOMA)

Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer. It frequently occurs in people with underlying liver illnesses such as hepatitis B or C, cirrhosis, or NAFLD/NASH (Forner, A. *et al.*, 2018). The incidence of HCC has been rising worldwide over the last 20 years and is expected to increase until 2030 in some countries, including the United States. The incidence of HCC is highest in Asia and Africa, where the endemic high prevalence of hepatitis B and hepatitis C strongly predisposes to the development of chronic liver disease and subsequent development of HCC (Asafo-Agyei, K. O., & Samant, H. 2023). Current international vaccination strategies for hepatitis B virus (HBV), and advances in the management of hepatitis C virus (HCV) infections, promise to have a major impact on the incidence of HCC, but their benefit will be realized slowly because of the very long latency period—20-30 years—from hepatic damage to HCC development.

1.3 ANATOMY OF LIVER

The liver is the body's largest gland, contributing to 2.5% of total weight. It performs multiple functions to maintain physiological homeostasis (Juza, R.M. and Pauli, E.M. 2014). The liver develops from foregut endoderm during the third week of gestation. The fetal liver receives blood via the portal and umbilical veins. The hepatic primordium is made up of cellular cords that form sinusoids connected by the left umbilical and portal veins, forming a continuous system. They form the right and left branches of the portal vein in adult livers. The capillary networks connect cranially to produce the right, middle, and left hepatic veins, which drain the liver to the inferior vena cava. The umbilical vein connects the left portal vein and the liver, transporting oxygenated blood from the placenta to the inferior vena cava (IVC) via the ductus venosus. The ductus venosus is present in the fetal circulation until shortly after birth, when it is obliterated to create the ligamentum

teres, which connects to the falciform ligament. The liver, weighing around 1500g in adults. The Glisson's capsule is a fibrous membrane that surrounds the region below the right and left diaphragm. Visceral peritoneum covers the liver both anteriorly and posteriorly, with the exception of a naked patch around the diaphragm. The diaphragm is supported by the coronary ligament, which extends to form the right and left triangular ligaments. The falciform ligament and gastrohepatic and hepatoduodenal ligaments support it anteriorly and at the porta hepatis, respectively. The hepatoduodenal ligament surrounds the porta hepatis, including the hepatic artery, portal vein, and extrahepatic bile ducts (House, M.G. *et al.*, 2006).

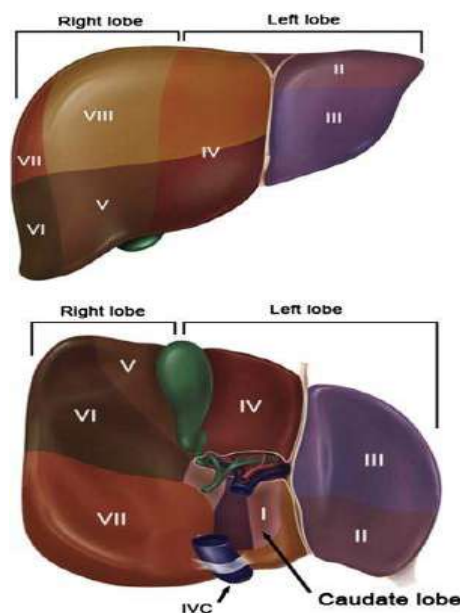


Fig:1.3 : Anterior and posterior surfaces of liver illustrating functional division of the liver into left and right hepatic lobes with Couinaud's segmental classification based on functional anatomy (House, M.G. *et al.*, 2006).

The liver is generally made of five types of specialized cells, which can be classified as parenchymal or nonparenchymal. At the cellular level, the liver parenchyma is structured in hexagonal lobules. The portal triad consists of branches from the hepatic artery, portal vein, and bile duct located near the lobule's perimeter (Si-Tayeb, K. *et al.*, 2010). The lobules feature thin-walled sinusoidal capillaries that drain the portal triad into a central vein. Sinusoidal capillaries are lined with specific endothelial cells that allow portal blood to connect

with hepatocytes. The majority of the liver is made up of hepatocytes, which form cords around sinusoidal capillaries (Malarkey, D.E. *et al.*, 2005). The liver's main functions include synthesis, storage, and filtering of portal venous blood (Kang, Y. *et al.*, 2023). Kupffer cells are specialized liver macrophages that perform immunological and phagocytic functions (Naito, M. *et al.*, 1997). Hepatic stellate cells, also known as perisinusoidal or Ito cells, play a crucial role in the pathological liver remodeling process associated with hepatic fibrosis, while being normally quiescent (Geerts, A. 2001). Bile duct epithelial cells transport bile and regulate its pH (Tietz, P.S. and LaRusso, N.F. 2006). The anatomy of the liver has undergone significant changes over the years. Historically, liver parenchyma was segmented based on topography, with the falciform ligament serving as the anatomical separation between the right and left hemilivers. In 1897, Sir James Cantlie's autopsy of a patient with an atrophic right lobe challenged the existing framework. Cantlie identified a line of atrophy lateral to the falciform ligament, going from the gallbladder fundus to the IVC as it exits the liver superiorly. This is now referred to as Cantlie's line (Van Gulik, T.M. and Van Den Esschert, J.W. 2010).

According to this taxonomy, the liver is separated into segments, sectors, and hemilivers in decreasing order of magnitude. Cantlie's line marks the continuation of the division between the right and left hemilivers. A region that is approximately surrounded by two hepatic veins or by a hepatic vein and the liver's margin is known as a hepatic sector. A section of the liver is a parenchyma that has its own biliary drainage system, independent vascular inflow, and outflow. Because of this, the liver is split into the right and left hemilivers at Cantlie's line, which roughly corresponds to the portal vein's bifurcation and the middle hepatic vein. The right posterior, right anterior, and left liver sectors are then formed by the division of the liver from lateral to medial by the right and middle hepatic veins. The ligamentum teres separated the left liver into a medial and lateral sector under Couinaud's method, and this division is still frequently made. The two-sector left hemiver has recently been replaced by a single, three-segment left sector (Bismuth, H. 2013). Because they can be identified during operative planning for right or left hepatectomies or bisegmentectomies, surgeons also refer to the divisions of the right, middle, and left hepatic veins as Glissonian pedicles. The hepatic artery and

cystic duct branches are part of the Glissonian pedicle, which is defined as the base of the branching vein. It is referred to as because Glisson's capsule has invested in it. With less blood lost during surgery, precise segmental dissection is possible when these pedicles are isolated and ligated.

1.3.1 *LIGAMENTOUS ATTACHMENTS*

An attachment that originates at or close to the umbilicus and extends along the umbilical fissure to the anterior portion of the liver is known as the falciform ligament. The anterior surface of the liver is where the falciform ligament runs cranially. It blends into the hepatic peritoneal lining and then travels posterosuperiorly to become the anterior part of the left and right coronary ligaments. Importantly from a surgical perspective, the hepatic veins empty into the inferior vena cava (IVC) near the base of the falciform ligament along the liver. (Raftery, A. 2007). The falciform ligament is frequently interpreted incorrectly to split the liver into the left and right lobes. This might be the case based on morphologic anatomy, however it is not the case in terms of function (explained below).

The ligamentum teres, also known as the round ligament, is located within the lower border of the falciform ligament. It is a piece of the obliterated umbilical vein (ductus venosus), which continues from the umbilicus into the umbilical fissure, where it joins the left branch of the portal vein in continuity with the ligamentum venosum. Between the left lobe anteriorly and the caudate lobe posteriorly on the inferior side of the liver, there is a fissure that contains the ligamentum venosum. The peritoneal folds of the smaller omentum (gastrohepatic ligament) also invest this cleft. The ductus venosus is in charge of diverting most of the umbilical vein's blood flow into the IVC throughout foetal life, delivering oxygenated blood from the placenta to the developing foetus. The umbilical vein shuts after delivery, marking the start of the newborn's physiological circulation. The umbilical vein may recanalize in the context of portal hypertension, allowing portasystemic collateralization through the abdominal wall, or caput medusae. A convex region without any peritoneum or ligamentous attachments can be found along the diaphragmatic surface of the liver at its cranial aspect. The thin fibroareolar tissue that covers this exposed portion of the liver connects it to the diaphragm. Adjacent

to the exposed portion of the liver, which is made up of the diaphragm's peritoneal reflections, is the coronary ligament. The left and right triangular ligaments are formed by the convergence of these regions to the left and right of the liver, respectively. The liver is attached to the retroperitoneum by the right coronary and right triangular ligaments, which run posteriorly and caudally towards the right kidney. Every attachment aids in fixing the liver in the abdomen's right upper quadrant. Sectioning these avascular linkages is necessary for the mobilisation of the liver during hepatic surgery. The liver is closely associated with numerous tissues and organs in upper abdominal surgery. IVC ligaments allow the IVC to remain in close proximity to the right hepatic lobe and caudate lobe (Kogure, K. *et al.*, 2007). The caval ligaments, which extend from the caudate and right hepatic lobe, are large membranous tissue bridges that form an extension of the Glisson capsule. These ligaments, which are crucial for surgery, are made of more than just connective tissue; they also include hepatocytes and the portal triads, two elements of the hepatic parenchyma. Hence, in order to prevent needless bleeding or bile leakage during hepatic surgery, these ligaments must be surgically managed during liver mobilisation.

1.3.2 PERIHEPATIC ORGANS/ANATOMY

There are several connections between the liver and the gastrointestinal tract. The gastrohepatic ligament, sometimes known as the upper side of the lesser omentum, is a connective tissue attachment that connects the left hepatic lobe to the stomach at the ligamentum venosum. It forms the connection between the two anatomical structures. The gastrohepatic ligament may contain important neurological and vascular structures, such as the vagus nerve's hepatic division and, if it exists, an aberrant left hepatic artery that originates in the left gastric artery. The right hepatic lobe is in close proximity, and occasionally in direct touch, with the colon's hepatic flexure, which marks the transition from the ascending to the transverse colon. Furthermore, the hepatoduodenal ligament (inferior aspect of the smaller omentum) and porta hepatis establish a direct contact between the duodenum and portal structures and the liver. Hepatic resection and related vascular and biliary repairs depend on anatomic comprehension of the portal anatomy. The common bile duct, hepatic artery, and portal vein all pass through the porta hepatis in a lateral, medial, and posterior pattern, respectively. The porta

hepatitis and hepato-pancreatico-biliary surgery are significantly impacted by the Winslow foramen, also known as the epiploic foramen. The foramen of Winslow is a passageway or link that connects the abdominal cavity and the smaller sac. It was first identified by Danish anatomist Jacob Winslow in 1732. A Pringle manoeuvre may be used to achieve total control of the hepatic vascular input during liver resection (Pringle, J.H. 1908).

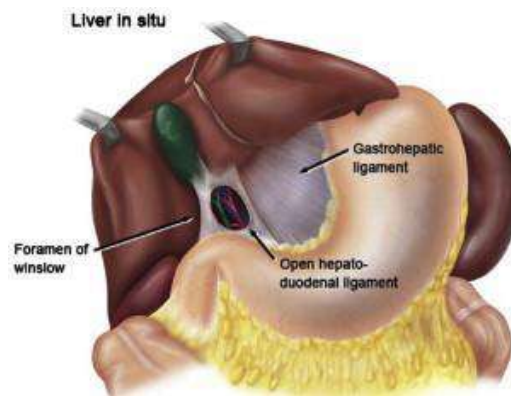


Fig:1.4 : Association of stomach, porta hepatis, and hepatic flexure to the Liver (Pringle, J.H. 1908).

The gallbladder is located near the posterior contact of segments IV and V in the gallbladder fossa. Through the cystic duct, it creates continuity with the common bile duct. Furthermore, the right hepatic artery is typically the parent vessel from which the cystic artery develops. Given the great anatomic heterogeneity of the biliary system, knowledge of the portal vasculature and anatomy is essential to prevent unintentional harm during any foregut, biliary, pancreatic, or hepatic surgery. Furthermore, the retroperitoneum, which is located beneath the right hepatic lobe, houses the right adrenal gland. Since the right adrenal vein empties directly into the IVC, caution should be exercised during hepatic mobilisation to prevent vein avulsion or unintentional dissection into the adrenal gland, which can cause severe bleeding.

1.3.3 LYMPHATIC AND NEURAL NETWORK

The lymph generated in the liver drains through the liver's superficial and deep lymphatic networks. Greater lymphatic drainage via the hepatic veins and portal vein branches towards the hilum and lateral phrenic nodes is the responsibility of the deep network (Skandalakis J.E. *et al.*, 2004). The Glisson capsule contains the

superficial network, which has surfaces on both the front and posterior sides. The anterior surface joins the mediastinal and internal mammary lymphatic networks by draining mostly through the bare portion of the liver to phrenic lymph nodes. The peripancreatic, pericardial, and celiac lymph nodes, as well as the cystic, common, and hepatic arteries, are among the hilar lymph nodes that the posterior surface network drains to. The surgical consequences of lymphadenectomy for pancreatic, liver, and gallbladder cancer are related to the lymphatic drainage patterns.

Liver function's neuronal innervation and regulation are intricate and poorly understood. The liver does, however, have sympathetic and parasympathetic neural innervation, just like the rest of the body. The vagi, right phrenic nerve, lower thoracic ganglia, and celiac plexus are the sources of nerve fibres. As the vagus nerves proceed from the thorax into the abdomen, they split into an anterior (left) and posterior (right) branch. The anterior vagus nerve is bifurcated into the cephalic and hepatic divisions. The hepatic division innervates the liver and is in charge of the parasympathetic innervation. It passes through the smaller omentum, or gastrohepatic ligament. The thoracic splanchnic nerves and the celiac plexus are the primary sources of sympathetic innervation (Skandalakis, J.E. *et al.*, 2004).

1.3.4 HEPATIC VASCULATURE

As the most vascular organ, the liver gets more than any other organ, up to 25% of the total cardiac output while it is at rest. Its dual blood supply is split specifically between the portal vein, which supplies 70% to 75% of the blood flow, and the hepatic artery, which supplies 25% to 30%. The hepatic venous system allows the arterial and portal blood to drain into the systemic circulation after they have finally mixed within the hepatic sinusoids (Blumgart, L.H. and Belghiti, J. 2007).

ARTERIAL VASCULATURE

This section discusses the most typical configurations of the liver's arterial vasculature, regardless of its variability. As seen in Fig. , the left gastric, splenic, and common hepatic arteries all arise from the celiac axis in the most common vascular configuration. The proper hepatic artery and the gastroduodenal artery split off from the common hepatic artery as it moves laterally. The gastroduodenal

artery has multiple indirect branches that lead to the pancreas and travels caudally to supply the proximal duodenum and pylorus. The right and left hepatic arteries, which supply the corresponding hepatic lobes, split off from the true hepatic artery as it passes through the medial portion of the hepatoduodenal ligament and the porta hepatis towards the liver.

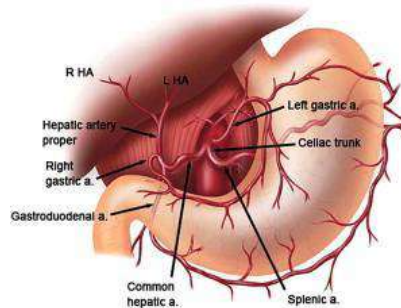


Fig:1.5 : Common hepatic arterial configuration. HA, hepatic artery (Blumgart, L.H. and Belghiti, J. 2007).

VENOUS VASCULATURE

The majority of the blood supply that gives the liver nutrition comes from the portal vein. The portal vein develops from the junction of the splenic and superior mesenteric veins beneath the pancreatic neck, as seen in above figure. The coronary (left gastric) vein, the cystic vein, and the tributaries of the right gastric and pancreaticoduodenal veins are further venous branches that empty into the portal vein. The portal vein is a low-pressure, valveless system that normally operates at pressures between 3 and 5 mm Hg. Given that it feeds the gastroesophageal variceal complex and develops into a significant portasystemic shunt in the event of portal hypertension, the coronary (left gastric) vein is particularly significant clinically.

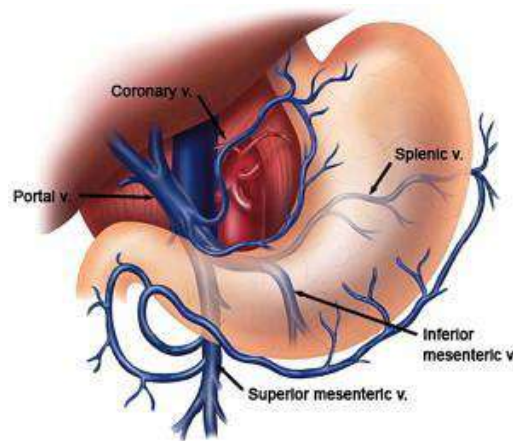


Fig:1.6 : Portal vein and the hepatic venous vasculature inflow
(Blumgart, L.H. and Belghiti, J. 2007).

1.3.5 BILIARY TREE

The ducts that make up the intrahepatic biliary tree, which usually follows the portal venous system, are in charge of forming and transferring bile from the liver to the duodenum. An anterior sectoral duct from segments V and VIII and a posterior sectoral duct from segments VI and VII combine to generate the right hepatic duct. Whereas the posterior duct travels in a lateral, horizontal direction, the anterior sectoral duct follows an anterior, vertical trajectory. Usually, the right duct has a brief extrahepatic course with variable branching. When performing surgery at the hilum of the liver, surgeons need to be aware of this changing anatomy. Draining the left liver, the left hepatic duct follows the left portal vein for a longer extrahepatic course and is less varied in its path. The common hepatic duct is formed by the union of the left and right hepatic ducts close to the hilar plate. The cystic duct joins the common hepatic duct as it runs caudally, creating the common bile duct. The common bile duct travels towards the pancreatic head within the lateral aspect of the hepatoduodenal ligament before emptying into the duodenum via the ampulla of Vater. The caudate lobe's biliary drainage varies, with both the left and right hepatic ducts showing signs of drainage.

1.4 ALCOHOL LIVER DISEASE

Alcohol liver disease (ALD) is a chronic and potentially fatal condition resulting from prolonged and excessive alcohol consumption (Rehm *et al.*, 2019). As one of the leading causes of liver-related morbidity and mortality worldwide, ALD poses a

significant public health challenge. The spectrum of ALD encompasses various pathological entities, ranging from hepatic steatosis to cirrhosis, each representing different stages of liver damage induced by alcohol abuse.

Chronic alcohol consumption initiates a cascade of molecular events within the liver, leading to oxidative stress, inflammation, and hepatocellular injury (Gao, B. and Bataller, R. 2011). Over time, these pathological changes can progress, ultimately resulting in irreversible liver damage and dysfunction. Despite advances in understanding ALD pathogenesis, effective treatments remain limited, highlighting the urgent need for novel therapeutic strategies.

Recognizing the clinical manifestations of ALD is crucial for early diagnosis and intervention. Patients with ALD may present with a wide array of symptoms, including fatigue, jaundice, abdominal pain, and signs of advanced liver disease such as ascites and hepatic encephalopathy. Timely diagnosis allows for the implementation of appropriate management strategies, including alcohol cessation, nutritional support, and pharmacotherapy, aimed at halting disease progression and improving patient outcomes. In conclusion, ALD represents a significant burden on individuals and healthcare systems globally, necessitating comprehensive approaches for prevention, diagnosis, and management. By addressing the multifactorial nature of ALD through evidence-based interventions, we can mitigate its impact on public health and improve the well-being of affected individuals.

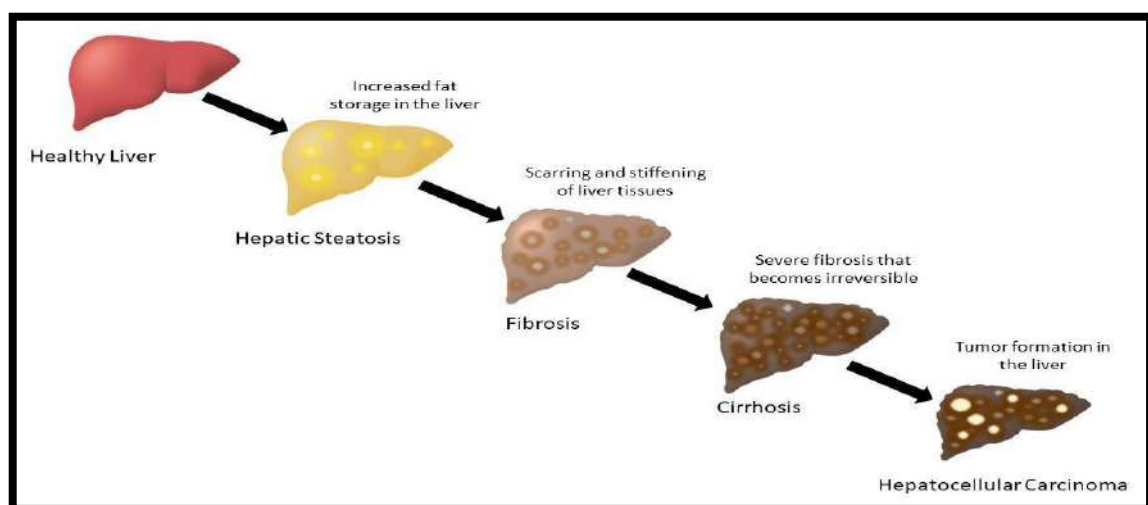


Fig:1.7 : Spectrum of Liver Disease (Gao, B. and Bataller, R. 2011)

Since the liver is where alcohol (ethanol) is mostly metabolised, alcohol consumption can cause early damage to it. Drinking alcohol continuously causes ALD through a number of lipid metabolism-related processes. Because ethanol oxidation disturbs normal lipid homeostasis, it increases lipid synthesis and storage in the liver. It accomplishes this by reducing the rate of autophagy in the liver as a result of lysosome biogenesis inhibition, which leads to a general decrease in lipid catabolism. Very low density lipoprotein, or VLDL, is typically produced by the liver to transfer triglycerides from the liver to peripheral organs. Alcohol, however, inhibits the liver's ability to export fat because it produces less intact VLDL. In effect, liver functionality is converted from breaking down lipids to storing lipids during alcohol exposure. These damaging effects of alcohol on lipid metabolism in the liver leads to the accumulation of fat and the development of alcohol-associated steatosis.

1.5 MECHANISM OF ALCOHOL

Alcohol, chemically known as ethanol, exerts its effects on the body through various mechanisms, impacting different organs and systems. Upon ingestion, alcohol is rapidly absorbed from the gastrointestinal tract into the bloodstream. Its effects are broad-ranging, affecting the central nervous system (CNS), cardiovascular system, liver, and other organs. Primarily, alcohol acts as a CNS depressant by enhancing the activity of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) and inhibiting the activity of the excitatory neurotransmitter glutamate. This dual action leads to the characteristic sedative and anxiolytic effects of alcohol, contributing to its use as a social lubricant and stress reliever. Additionally, alcohol affects other neurotransmitter systems, including dopamine and serotonin, which are involved in the regulation of mood and reward, thereby influencing the reinforcing properties of alcohol consumption. (Crews, F.T. and Vetreno, R.P. 2015).

In the cardiovascular system, moderate alcohol consumption has been associated with a lower risk of coronary artery disease, primarily due to its ability to increase levels of high-density lipoprotein (HDL) cholesterol and reduce platelet aggregation. However, excessive alcohol consumption can lead to hypertension, arrhythmias, and cardiomyopathy, increasing the risk of cardiovascular

events. Chronic alcohol consumption exerts profound effects on the liver, leading to the development of fatty liver, alcoholic hepatitis, fibrosis, and cirrhosis. Alcohol metabolism primarily occurs in the liver through oxidation by alcohol dehydrogenase (ADH) and cytochrome P450 enzymes, resulting in the formation of toxic by-products such as acetaldehyde. Chronic exposure to acetaldehyde and oxidative stress contributes to liver inflammation, fibrosis, and ultimately liver failure. Moreover, alcohol consumption can impair gastrointestinal function, increase the permeability of the intestinal mucosa, and disrupt the gut microbiota composition, contributing to conditions such as alcoholic gastritis, pancreatitis, and increased susceptibility to infections.

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the CNS. Alcohol enhances GABAergic neurotransmission by increasing the activity of GABA receptors (GABAA receptors), leading to an overall inhibitory effect on the brain. This increase in inhibition contributes to alcohol's sedative and anxiolytic effects. Glutamate is the primary excitatory neurotransmitter in the brain. Alcohol inhibits the activity of N-methyl-D-aspartate (NMDA) receptors, which are glutamate receptors. By doing so, alcohol dampens glutamatergic neurotransmission, resulting in sedation and cognitive impairment. Alcohol increases the release of dopamine in the brain's reward pathway, primarily in the nucleus accumbens. This increase in dopamine release is associated with feelings of pleasure and reward, contributing to the reinforcing effects of alcohol consumption. Alcohol also affects the serotonin neurotransmitter system. Chronic alcohol consumption can lead to alterations in serotonin levels and function, which may contribute to mood disturbances, including depression and anxiety. Endorphins are neurotransmitters that act as natural painkillers and contribute to feelings of well-being and euphoria. Alcohol consumption triggers the release of endorphins, which can produce pleasurable effects and contribute to the reinforcing properties of alcohol.

Alcohol can also influence various neuropeptide systems in the brain, including the opioid and neuropeptide Y systems. These systems are involved in modulating stress responses, mood regulation, and reward processing, contributing to alcohol's effects on emotions and behavior. Alcohol metabolism leads to the production of acetaldehyde, a toxic by-product. Acetaldehyde is further

metabolized into acetate by the enzyme aldehyde dehydrogenase. Acetate can then be used as an energy source in the brain. However, acetaldehyde accumulation may contribute to some of alcohol's negative effects, including hangover symptoms. Chronic alcohol consumption can lead to neuroadaptive changes in the brain, including alterations in neurotransmitter levels, receptor sensitivity, and neuronal function. These changes may contribute to the development of tolerance, dependence, and addiction to alcohol.

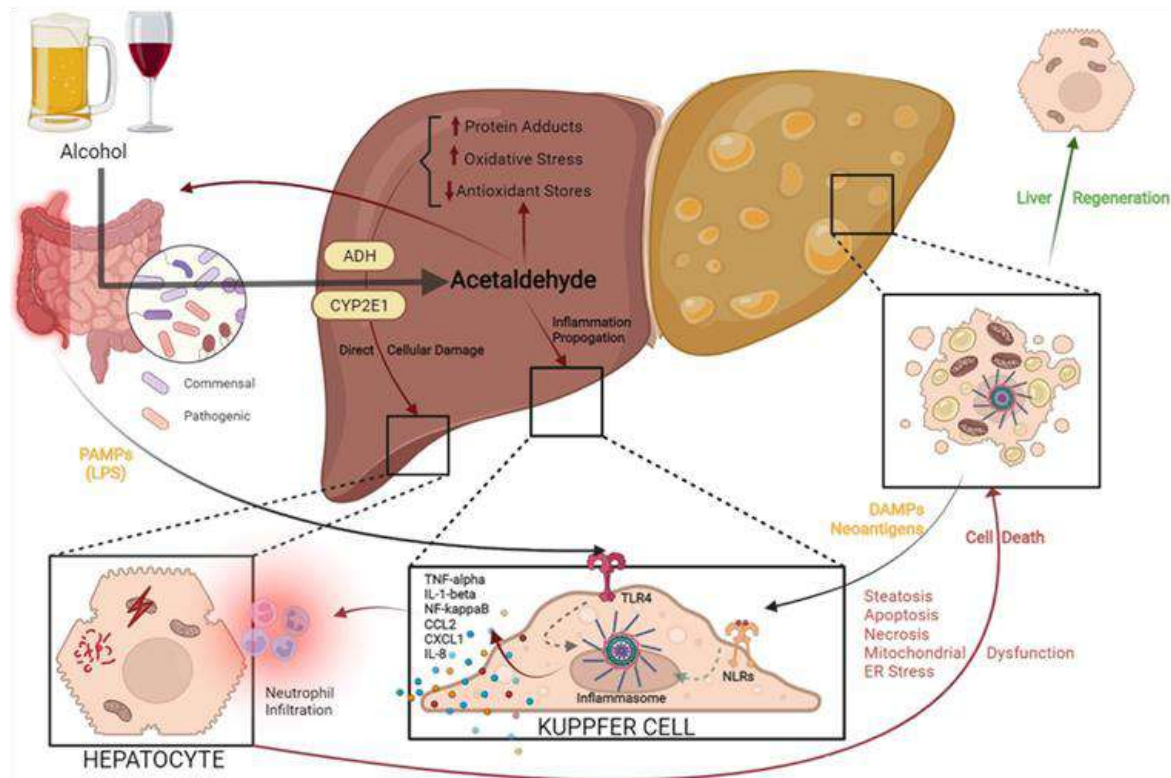


Fig:1.8 : Pathophysiology of alcohol-induced liver damage alcohol consumption (Ratan, P. et al., 2022).

1.6 TOXICITY OF ALCOHOL:

Alcohol, while commonly consumed in many societies, can have significant toxic effects on the human body when consumed in excessive quantities or over prolonged periods. Ethanol, the active ingredient in alcoholic beverages, is a central nervous system depressant that affects various organs and systems throughout the body. The toxicity of alcohol stems from its ability to disrupt the normal functioning of cells and tissues, leading to a wide range of detrimental health effects.

One of the most immediate and well-known toxic effects of alcohol is its impact on the brain. Alcohol acts as a depressant on the central nervous system, slowing down neurotransmission and altering brain chemistry. This can result in impaired judgment, coordination, and cognitive function, leading to increased risk of accidents, injuries, and even alcohol-induced blackouts. Chronic alcohol abuse can also lead to long-term neurological damage, including memory loss, cognitive impairment, and an increased risk of neurological disorders such as dementia. National Institute on Alcohol Abuse and Alcoholism (NIAAA).

Furthermore, alcohol exerts toxic effects on the liver, a vital organ responsible for metabolizing and detoxifying harmful substances in the body. Chronic alcohol consumption can cause liver inflammation, fatty liver disease, and ultimately progress to more severe conditions such as alcoholic hepatitis, fibrosis, and cirrhosis. These conditions can significantly impair liver function and may ultimately lead to liver failure, a life-threatening condition requiring medical intervention, or transplantation.

Alcohol also has detrimental effects on the cardiovascular system, contributing to the development of hypertension, irregular heart rhythms, and an increased risk of heart disease and stroke. Chronic alcohol abuse can weaken the heart muscle, leading to conditions such as alcoholic cardiomyopathy, which can result in heart failure. World Health Organization (WHO) Additionally, alcohol can raise levels of triglycerides and promote the formation of fatty deposits in the arteries, increasing the risk of atherosclerosis and coronary artery disease.

Moreover, alcohol consumption can damage the gastrointestinal tract, causing inflammation of the esophagus (esophagitis), stomach (gastritis), and pancreas (pancreatitis). These conditions can lead to symptoms such as abdominal pain, nausea, vomiting, and gastrointestinal bleeding. Prolonged alcohol abuse is also associated with an increased risk of gastrointestinal cancers, including those of the esophagus, stomach, liver, and pancreas.

In addition to its direct toxic effects on organs and systems, alcohol misuse can also contribute to various social, psychological, and behavioral problems, including addiction, depression, anxiety, and relationship issues. The impact of alcohol toxicity extends beyond the individual to affect families, communities, and society

as a whole, contributing to significant healthcare costs, lost productivity, and social harm.

To mitigate the harmful effects of alcohol toxicity, public health efforts focus on raising awareness, promoting responsible drinking habits, and providing support and resources for those struggling with alcohol abuse or addiction. Centers for Disease Control and Prevention (CDC) Strategies include education campaigns, regulations on alcohol advertising and availability, screening and intervention programs, as well as access to treatment and support services for individuals and families affected by alcohol-related problems.

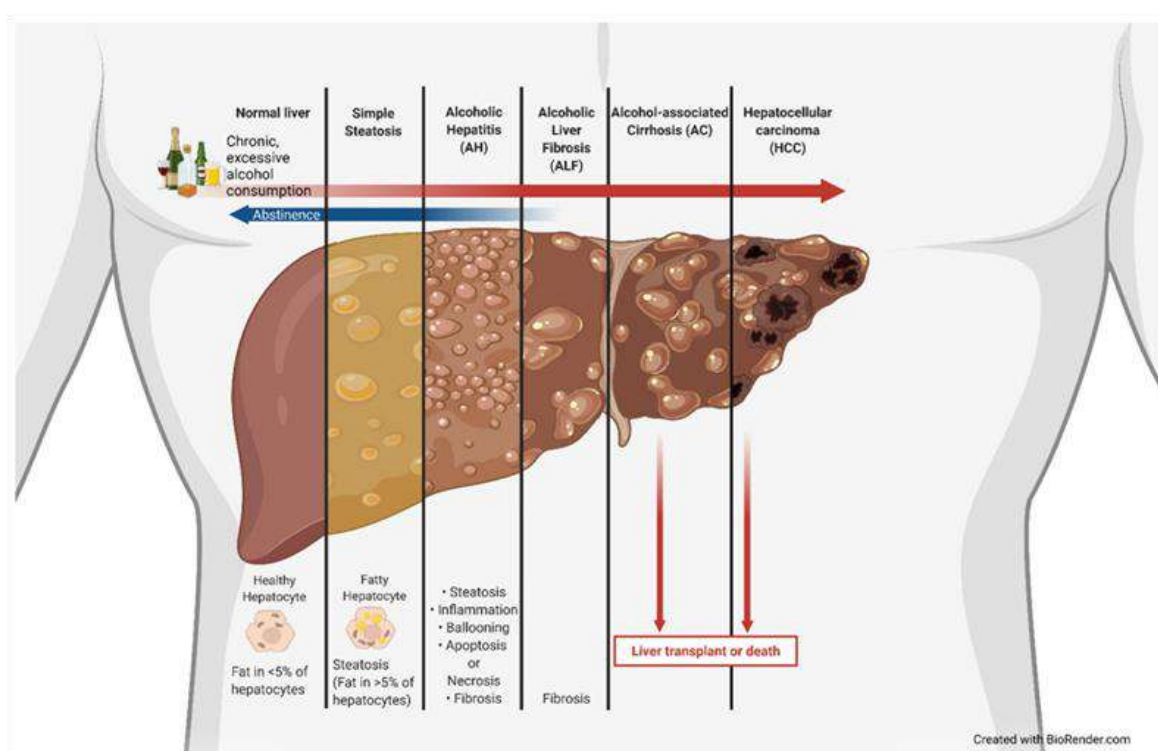


Fig:1.9 : Alcohol toxicity (Way, G.W et al., 2022)

1.7 ALD STATISTICS IN WORLD & INDIA

Over half of the people in the USA, Europe, and the Western Pacific drink alcohol, and the Global Status Report on Alcohol and Health 2018 estimates that there are currently 2.3 billion drinkers globally. Many disorders, including acute pancreatitis, alcohol-related cardiomyopathy, and alcohol-related liver diseases (ARLD), have a chronic heavy drinking history as its aetiology or risk factor (Niu, X. et al., 2023). According to a study, the number of alcohol poisoning deaths in the USA increased by 2.1 times between 2000 and 2019. The World Health Organisation

estimates that approximately 3 million fatalities worldwide in 2016 were related to drinking. Of all the diseases with the highest proportion, alcohol-related digestive disorders were responsible for 21.3% of the deaths. There were 637,000 deaths in all, 607,000 of which were related to ARLD. As a result, one of the main causes of alcohol-related mortality is now ARLD (Parker, R. *et al.*, 2023). The role of HBV as the primary cause of chronic liver disease is gradually waning due to the widespread use of antiviral medication and the hepatitis B vaccine, while attention to alcohol consumption is rising. According to a study, there was a 3.73% increase in the worldwide prevalence of chronic liver disorders caused by alcohol use between 2005 and 2015. In the USA, the population with cirrhosis had a 43% rise in the prevalence of alcoholic liver cirrhosis in just 7 years. Between 2000 and 2009, the number of ARLD deaths in South Korea rose from 1403 to 3588 (Bang, H.-A. *et al.*, 2015). These investigations revealed a progressive increase in the ARLD burden. Therefore, developing appropriate preventative and control programmes requires an understanding of the epidemiology of ARLD.

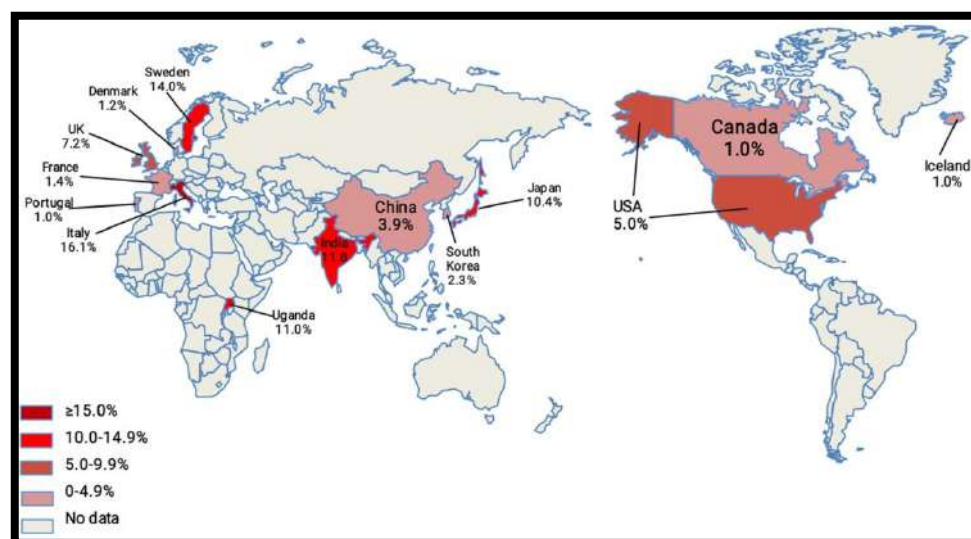


Fig:1.10 : The prevalence of ARLD worldwide. The level of red represented the predominance in 14 different countries. Portugal, Canada, Iceland, France, China, USA, Denmark, South Korea, Uganda, India, UK, Sweden, Japan, and Italy were among the 14 nations (Bang, H.-A. *et al.*, 2015).

Alcohol raises the risk of mortality from cardiovascular disease by 3.2 times, cancer by 5.1 times, and liver disease-related mortality by 260 times. Almost 60% of cirrhosis cases in Europe, North America, and Latin America are caused by alcohol, which is also the primary cause of cirrhosis worldwide. Additionally, about

35% of individuals with AUD may develop various kinds of alcohol-associated liver disease (ALD). In actuality, a country or region's percentage of cirrhosis is positively correlated with higher rates of heavy alcohol intake. The global prevalence of alcohol intake and ALD is shown in Fig. 1.10.

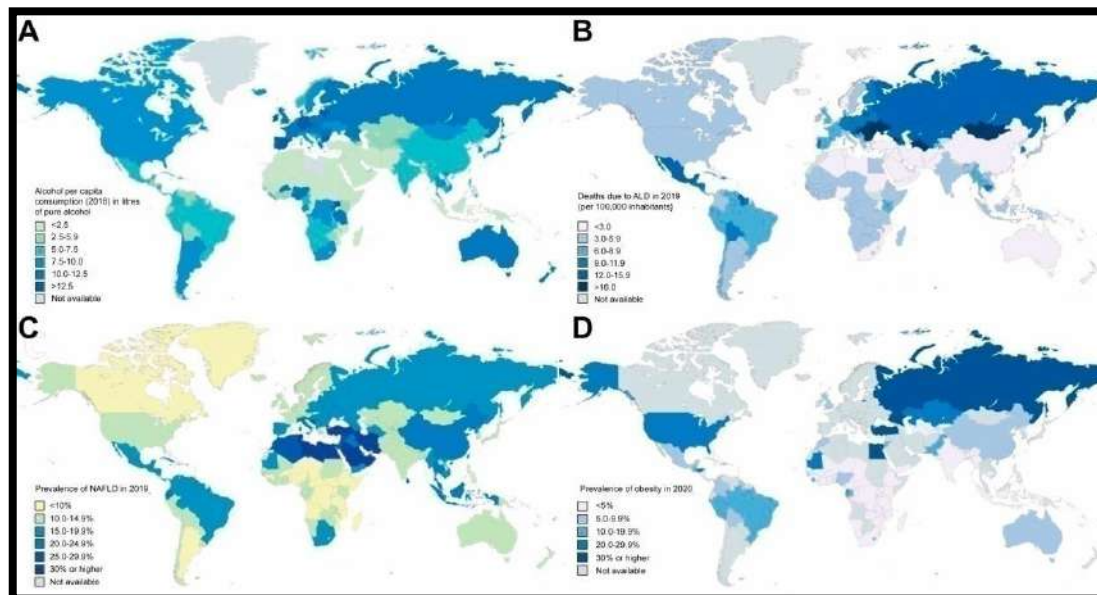


Fig:1.11 : Worldwide frequency of ALD, NAFLD, obesity and alcohol use. NAFLD stands for non-alcoholic fatty liver disease; ALD stands for alcohol-associated liver disease (Hagstrom, H. et al., 2018).

Alcohol-associated hepatitis has become more common worldwide in recent years, particularly among women and young people. The majority of the illness burden affects individuals between the ages of 15 and 44, primarily young people who are in their prime working years. This is consistent with the finding that regular drinking raises the risk of alcohol-associated cirrhosis in late adolescence (Hagstrom, H. et al., 2018). About 22.36% of deaths in people of all ages from liver cirrhosis and other chronic liver diseases in 2019 were connected to hepatitis B. Alcohol consumption contributed to 24.90% of these deaths, while hepatitis C was responsible for 26.75%. Of the deaths, 9.20% were due to non-alcoholic steatohepatitis (NASH), with the remaining 16.78% being caused by other causes. In particular, hepatitis B (COHB)-related liver cirrhosis and other chronic liver disorders were responsible for 331,266 (278,542–392,126) fatalities in 2019, a 14.36% increase from 1990 [289,682 (249,026–331,355)]. But during this time, the ASMR for hepatitis B-related liver cirrhosis and other chronic liver diseases

dropped by 42.10%, from 6.96 (6.00-7.95) per 100,000 people in 1990 to 4.03 (3.39–4.76) per 100,000 people in 2019. HBV infection is now the third most common cause of ASMR for these conditions (Wu, X.-N. *et al.*, 2024).

1.7.1 IN INDIA:

In India, liver disease is becoming epidemic scale, affecting one in five adults. The shocking number of liver-related deaths in India has reached 268,580 (3.17% of all deaths) annually, accounting for 18.3% of the 2 million liver-related deaths worldwide.

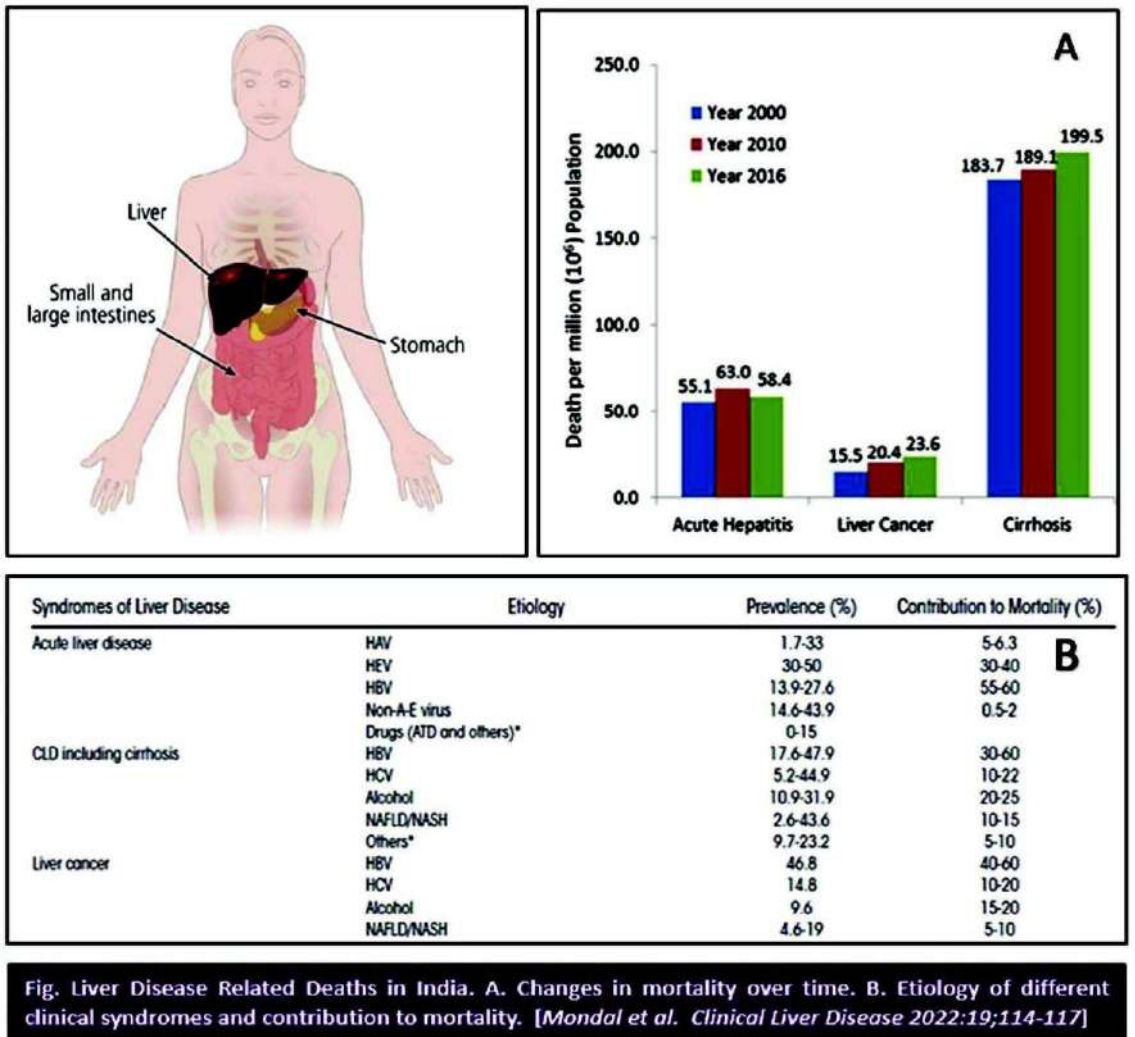


Fig:1.12 : Liver Disease related deaths in India (Greater Kashmir Paper)

The statistics indicating a rise in liver disease deaths in India since 1980 is concerning, as it differs from China where the trend has been stagnant or even declining (Figure represents from Greater Kashmir Paper). India's healthcare

system and economy have been severely impacted by liver disease. India stands out in that it includes a number of tropical diseases that impact the liver and play an important part in the narrative of illness, disability, and death.

A wide range of liver diseases are on the rise in India as a result of the country's current cultural and lifestyle shift, which includes a gradual adoption of a western diet, sedentary lifestyles, and a general lack of long-standing taboos surrounding alcohol. This shift involves the general importance of nonalcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) as liver disease causes rising above and beyond viral causative elements.

1.8 ALCOHOLIC LIVER DISEASE IN PRECLINICAL STUDIES

Alcoholic liver disease (ALD) remains a significant global health concern, characterized by a spectrum of liver disorders ranging from steatosis (fatty liver) to hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. Chronic alcohol consumption disrupts various metabolic pathways in the liver, leading to inflammation, oxidative stress, and ultimately, liver damage. Preclinical studies, conducted primarily in animal models, play a crucial role in elucidating the underlying mechanisms of ALD and developing potential therapeutic interventions. Alcoholic liver disease encompasses a range of liver pathologies caused by chronic alcohol consumption. It is a leading cause of liver-related morbidity and mortality worldwide. ALD typically progresses through several stages, starting with hepatic steatosis, which involves the accumulation of fat in hepatocytes. Continued alcohol abuse can lead to inflammation (alcoholic hepatitis), fibrosis, cirrhosis, and in some cases, hepatocellular carcinoma. Preclinical studies investigating ALD often utilize animal models to mimic the disease's progression and study underlying mechanisms. These models offer valuable insights into the pathophysiology of ALD and provide a platform for testing potential therapeutic interventions. Commonly used animal models include: Rodent Models and Non-Rodent Models. Ethanol Liquid Diet Models Rodents are fed with liquid diets containing ethanol to induce ALD-like symptoms. These models allow for precise control over ethanol intake and duration of exposure. Intermittent exposure to high doses of ethanol mimics binge drinking patterns observed in humans, leading to acute liver injury and inflammation. Genetically engineered mice with alterations in

genes involved in alcohol metabolism or liver function are used to study specific aspects of ALD pathogenesis (Gao, B. 2012). Non-Rodent Models include Pigs and Zebrafish Models. Pigs share physiological and metabolic similarities with humans, making them valuable models for studying ALD (Louvet, A. and Mathurin, P. 2015). Zebrafish offer advantages such as optical transparency and genetic tractability, facilitating the study of ALD pathogenesis and screening of potential therapeutics.

1.8.1 MECHANISM OF ALCOHOLIC LIVER DISEASE

Preclinical studies have elucidated various molecular and cellular mechanisms underlying ALD development and progression:

Hepatic Steatosis

Chronic alcohol consumption disrupts lipid metabolism, leading to the accumulation of triglycerides in hepatocytes. Activation of sterol regulatory element-binding proteins (SREBPs) and inhibition of peroxisome proliferator-activated receptor alpha (PPAR α) contribute to lipid accumulation.

Oxidative Stress and Inflammation

Ethanol metabolism generates reactive oxygen species (ROS), causing oxidative stress and lipid peroxidation. Activation of innate immune responses, including Kupffer cells and neutrophils, triggers hepatic inflammation and cytokine production.

Fibrosis and Cirrhosis

Persistent liver injury and inflammation promote activation of hepatic stellate cells (HSCs) and deposition of extracellular matrix proteins, leading to fibrosis. Progression to cirrhosis is characterized by extensive fibrosis, vascular remodeling, and loss of liver function (You, M. and Arteel, G.E. 2019).

1.8.2 THERAPEUTIC TARGETS AND INTERVENTIONS

Preclinical studies have identified several potential therapeutic targets for ALD treatment:

Antioxidants

Compounds with antioxidant properties, such as N-acetylcysteine (NAC) and vitamin E, have shown promise in reducing oxidative stress and mitigating liver injury in animal models.

Anti-inflammatory Agents

Inhibitors of pro-inflammatory cytokines (e.g., TNF-alpha) and chemokine receptors have demonstrated efficacy in attenuating hepatic inflammation and improving liver histology.

Modulators of Lipid Metabolism

Agents targeting lipid metabolism pathways, including SREBP inhibitors and PPAR α agonists, hold potential for reducing hepatic steatosis in ALD.

Fibrosis Modulators

Antifibrotic compounds, such as inhibitors of HSC activation or extracellular matrix deposition, have shown efficacy in preventing or reversing liver fibrosis in preclinical models (Szabo, G. and Petrasek, J. 2015).

ALCOHOLIC LIVER DISEASE IN ANIMAL MODELS

Rodent Models

Rodent models, particularly mice and rats, have been extensively utilized in ALD research due to their genetic manipulability, cost-effectiveness, and similarities to human physiology and pathology. Various models have been developed to mimic different stages of ALD, including chronic ethanol feeding, binge drinking, and intragastric infusion of ethanol. These models replicate key features of human ALD, such as hepatic steatosis, inflammation, and fibrosis, allowing researchers to study disease progression and test therapeutic interventions.

Non-Human Primate Models

Non-human primates, such as chimpanzees and baboons, share genetic and physiological similarities with humans, making them valuable models for studying complex diseases like ALD. While less commonly employed due to ethical and logistical constraints, non-human primate models offer unique advantages,

including the ability to study long-term effects of alcohol exposure and to assess interindividual variability in disease susceptibility and response to treatment.

Zebrafish Models

Zebrafish have emerged as a powerful model organism for studying liver development and disease due to their optical transparency, rapid development, and amenability to genetic manipulation. Although zebrafish do not naturally consume alcohol, exposure to ethanol solutions can induce hepatic steatosis and liver injury, making them a valuable tool for investigating the molecular mechanisms underlying ALD pathogenesis.

1.9.1 PATHOPHYSIOLOGY OF ALD IN ANIMAL MODELS

1.9.1.1 STEATOSIS

Hepatic steatosis, characterized by the accumulation of triglycerides within hepatocytes, is a hallmark feature of ALD. Animal models have elucidated several mechanisms contributing to alcohol-induced steatosis, including alterations in lipid metabolism, increased fatty acid uptake, and impaired mitochondrial function (Zhu, L. *et al.*, 2022). Rodent models have been instrumental in identifying key regulators of hepatic lipid homeostasis, such as peroxisome proliferator-activated receptor alpha (PPAR α) and sterol regulatory element-binding protein 1c (SREBP-1c), and their dysregulation in response to alcohol exposure.

1.9.1.2 INFLAMMATION

Inflammation plays a pivotal role in the progression of ALD, driving the transition from simple steatosis to more severe forms of liver injury. Animal models have revealed intricate crosstalk between immune cells, cytokines, and gut-derived microbial products in orchestrating hepatic inflammation in response to alcohol (Mathews, S. *et al.*, 2014). Activation of toll-like receptors (TLRs) by gut-derived endotoxins triggers the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) and interleukin-1 beta (IL-1 β), leading to hepatocyte injury and recruitment of inflammatory cells to the liver (Mandrekar, P. and Szabo, G. 2009).

1.9.1.3 FIBROSIS AND CIRRHOSIS

Persistent inflammation and hepatocyte injury can culminate in the development of hepatic fibrosis, characterized by excessive deposition of extracellular matrix proteins such as collagen. Animal models have been instrumental in elucidating the cellular and molecular mechanisms driving alcohol-induced fibrogenesis, including activation of hepatic stellate cells (HSCs) and dysregulated production of profibrotic cytokines such as transforming growth factor-beta (TGF- β) (Galicía-Moreno, M. and Gutiérrez-Reyes, G. 2014). Long-term alcohol feeding studies in rodents have demonstrated progressive fibrosis and cirrhosis, recapitulating key features of advanced ALD observed in humans (Lieber, C.S. 2000).

1.9.2 THERAPEUTIC IMPLICATIONS AND FUTURE DIRECTIONS

1.9.2.1 TARGETING STEATOSIS

Given the central role of hepatic steatosis in ALD pathogenesis, therapeutic strategies aimed at reducing lipid accumulation represent promising avenues for intervention. Pharmacological agents targeting lipid metabolism pathways, such as PPAR α agonists and AMP-activated protein kinase (AMPK) activators, have shown efficacy in preclinical studies using animal models of ALD (Rao, R.K. *et al.*, 2004). Moreover, dietary interventions, including supplementation with omega-3 fatty acids and choline, have been shown to attenuate alcohol-induced steatosis in rodents, highlighting the potential for nutritional interventions in ALD management.

1.9.2.2 MODULATING INFLAMMATION AND FIBROSIS

Inhibition of inflammatory pathways and fibrogenic signaling represents another therapeutic approach for ALD. Animal studies have demonstrated the efficacy of anti-inflammatory agents such as corticosteroids and inhibitors of TLR signaling in ameliorating alcohol-induced liver injury. Furthermore, targeting profibrotic mediators such as TGF- β and platelet-derived growth factor (PDGF) has shown promise in attenuating hepatic fibrosis in preclinical models (Mandrekar, P. and Szabo, G. 2009). However, the translation of these findings into effective therapies for human ALD remains a challenge, emphasizing the need for further research to validate therapeutic targets and optimize treatment strategies.

1.9.2.3 PERSONALIZED MEDICINE APPROACHES

The heterogeneity in ALD progression and treatment response underscores the importance of personalized medicine approaches tailored to individual patient characteristics. Animal models offer valuable tools for studying inter individual variability in disease susceptibility and treatment response, allowing for the development of precision therapies for ALD. Integrating genetic, epigenetic, and environmental factors in preclinical models can facilitate the identification of biomarkers predictive of disease progression and treatment outcomes, ultimately guiding personalized treatment strategies in clinical practice. Animal models have been indispensable in advancing our understanding of ALD pathophysiology and in the development of therapeutic interventions. By recapitulating key features of human disease, these models have provided valuable insights into the mechanisms underlying ALD progression and have facilitated the identification of novel therapeutic targets. Continued research using animal models, coupled with translational studies in humans, holds promise for the development of effective treatments for ALD and ultimately improving patient outcomes.

1.10 ZEBRAFISH MODEL

Zebrafish (*Danio rerio*) has emerged as a powerful model organism in biomedical research due to its numerous advantages such as high fecundity, transparent embryos, and genetic tractability. Initially utilized in developmental biology, the zebrafish model has expanded into various fields including neurobiology, toxicology, and drug discovery. This review provides a comprehensive examination of the zebrafish model, highlighting its contributions to scientific advancements and the elucidation of biological mechanisms across diverse disciplines.

1.10.1 DEVELOPMENTAL BIOLOGY AND GENETICS

Zebrafish embryos develop externally, allowing for easy observation of embryogenesis under the microscope. The transparency of zebrafish embryos enables real-time visualization of developmental processes such as gastrulation, organogenesis, and tissue morphogenesis. Additionally, the rapid development from embryo to larval stage facilitates high-throughput screening assays for studying developmental abnormalities and genetic mutations (Kimmel *et al.*, 1995).

Forward genetic screens in zebrafish have led to the identification of key genes involved in vertebrate development, including the establishment of left-right asymmetry (Supp *et al.*, 1997).

1.10.2 NEUROBIOLOGY AND BEHAVIOR

Zebrafish possess a complex nervous system with homologous brain regions to mammals, making them an ideal model for studying neurodevelopment and behavior. The optical clarity of zebrafish larvae permits non-invasive imaging techniques such as two-photon microscopy to investigate neuronal activity *in vivo* (Ahrens, M.B. *et al.*, 2013). Zebrafish exhibit a wide range of behaviours including locomotion, social interaction, and learning, making them suitable for behavioural studies (Lin, Q. *et al.*, 2015). Mutant zebrafish lines with neuro developmental disorders provide valuable insights into the genetic basis of neurological diseases (Norton, W. and Bally-Cuif, L. 2010).

1.10.3 DRUG DISCOVERY AND TOXICOLOGY

The zebrafish model offers a cost-effective platform for drug discovery and toxicity testing. Zebrafish embryos are permeable to small molecules, allowing for high-throughput chemical screens to identify potential therapeutic compounds (Peterson, R.T. *et al.*, 2000). Moreover, the conservation of drug metabolism pathways between zebrafish and humans enables the prediction of drug metabolism and toxicity (Parng *et al.*, 2002). Zebrafish larvae are commonly used in toxicological studies to assess the effects of environmental pollutants and pharmaceuticals on embryonic development and organ function (Incardona, J.P. *et al.*, 2004).

1.10.4 CANCER RESEARCH

Zebrafish models of cancer offer unique opportunities to study tumor initiation, progression, and metastasis *in vivo*. Transgenic zebrafish lines expressing fluorescent reporters under the control of tissue-specific promoters facilitate the visualization of tumor growth and metastatic spread (White *et al.*, 2008). Zebrafish xenograft models, where human cancer cells are transplanted into zebrafish embryos, enable rapid assessment of tumor response to chemotherapy drugs (Lee *et al.*, 2005). Zebrafish cancer models have been instrumental in identifying

novel oncogenes and tumor suppressor genes involved in various cancer types (Langenau *et al.*, 2007).

1.10.5 CARDIOVASCULAR BIOLOGY

Zebrafish possess a fully functional cardiovascular system that shares similarities with mammals, making them an attractive model for studying heart development and disease. Forward genetic screens in zebrafish have identified key regulators of cardiac morphogenesis and function (Stainier *et al.*, 1996). Zebrafish mutants with cardiovascular defects provide insights into the genetic basis of congenital heart diseases (Beis *et al.*, 2005). Furthermore, zebrafish embryos are amenable to high-resolution imaging techniques such as confocal microscopy, allowing for detailed visualization of cardiac structure and function (Bakkers, 2011).

1.10.6 REGENERATIVE MEDICINE

Zebrafish exhibit remarkable regenerative abilities, particularly in organs such as the heart, fins, and central nervous system. Studying the molecular mechanisms underlying zebrafish regeneration may provide insights into promoting tissue repair in humans. Zebrafish models of heart regeneration have identified key signaling pathways involved in cardiomyocyte proliferation and tissue remodeling (Poss *et al.*, 2002). Moreover, the ability to perform genetic screens in zebrafish allows for the identification of genes essential for tissue regeneration (Johnson and Weston, 1995). In conclusion, the zebrafish model has revolutionized biomedical research across various disciplines including developmental biology, neurobiology, drug discovery, cancer research, cardiovascular biology, and regenerative medicine. Its amenability to genetic manipulation, optical transparency, and physiological relevance to humans make it an invaluable tool for studying complex biological processes and disease mechanisms. Continued advancements in zebrafish research hold great promise for the discovery of novel therapeutic strategies and the advancement of precision medicine.

1.11 ADULT ZEBRAFISH MODEL IN ALCOHOLIC LIVER DISEASE

Alcoholic liver disease (ALD) remains a significant global health concern, characterized by liver inflammation, steatosis, fibrosis, and cirrhosis, primarily resulting from chronic alcohol consumption. While various animal models have

been employed to study ALD, the adult zebrafish (*Danio rerio*) has emerged as a promising model due to its genetic tractability, regenerative capacity, and physiological similarities to humans. This review aims to explore the utilization of the adult zebrafish model in elucidating the pathogenesis of ALD and evaluating potential therapeutic interventions.

1.11.1 ZEBRAFISH AS A MODEL ORGANISM

Zebrafish possess several advantages as a model organism for studying liver diseases, including ALD. Their external fertilization, rapid embryonic development, and transparent embryos facilitate real-time observation of organ development and pathophysiological processes. Moreover, zebrafish share substantial genetic homology with humans, with approximately 70% of human genes having orthologs in zebrafish, making them valuable for translational research (Howe *et al.*, 2013).

1.11.2 ESTABLISHMENT OF ALD IN ZEBRAFISH

To induce ALD in zebrafish, chronic ethanol exposure protocols have been developed, mimicking human alcohol consumption patterns. Typically, adult zebrafish are exposed to ethanol through immersion in water supplemented with ethanol. This method allows for precise control over ethanol concentrations and exposure durations, enabling the study of various stages of ALD progression, from early steatosis to advanced fibrosis and cirrhosis (Passeri *et al.*, 2015).

1.11.3 PATHOPHYSIOLOGICAL FEATURES OF ALD IN ZEBRAFISH

Studies utilizing the adult zebrafish model have elucidated key pathophysiological features of ALD, including hepatocyte injury, inflammation, oxidative stress, and fibrogenesis. For instance, ethanol exposure in zebrafish leads to hepatocyte apoptosis, as evidenced by increased expression of apoptotic markers such as caspases and TUNEL-positive cells (Choi *et al.*, 2014). Additionally, ethanol-induced inflammation in zebrafish liver is characterized by upregulation of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF- α) and interleukin-1 beta (IL-1 β) (Zhang *et al.*, 2014).

1.11.4 ROLE OF LIPID METABOLISM IN ALD PROGRESSION

Dysregulation of lipid metabolism is a hallmark of ALD, contributing to the development of hepatic steatosis and subsequent liver injury. Zebrafish models

have provided insights into the mechanisms underlying ethanol-induced lipid accumulation in the liver. Ethanol exposure disrupts lipid metabolism pathways in zebrafish, leading to increased hepatic triglyceride levels and lipid droplet accumulation (Choi *et al.*, 2015). Moreover, studies have identified alterations in the expression of genes involved in fatty acid synthesis, oxidation, and transport in ethanol-exposed zebrafish, highlighting the role of lipid dysregulation in ALD pathogenesis (Passeri *et al.*, 2017).

1.11.5 INFLAMMATORY SIGNALING PATHWAYS IN ALD

Inflammation plays a pivotal role in the progression of ALD, contributing to tissue damage and fibrogenesis. The zebrafish model has been instrumental in deciphering the inflammatory signaling pathways activated by ethanol exposure. Notably, nuclear factor-kappa B (NF- κ B) signaling has been implicated in ethanol-induced liver inflammation in zebrafish, with studies demonstrating increased NF- κ B activity and expression of its target genes in ethanol-treated zebrafish larvae (Choi *et al.*, 2016). Furthermore, inhibition of NF- κ B signaling attenuates ethanol-induced liver injury and inflammation in zebrafish, highlighting its therapeutic potential (Kim *et al.*, 2019).

1.11.6 FIBROGENESIS AND CIRRHOSIS IN ZEBRAFISH ALD MODELS

Progression to advanced stages of ALD, such as fibrosis and cirrhosis, involves the activation of hepatic stellate cells (HSCs) and excessive extracellular matrix deposition. Zebrafish models have provided valuable insights into the cellular and molecular mechanisms underlying fibrogenesis in ALD. Ethanol exposure activates HSCs in zebrafish liver, leading to increased expression of fibrogenic markers like alpha-smooth muscle actin (α -SMA) and collagen (Tsedensodnom *et al.*, 2013). Moreover, studies have identified key signaling pathways, including transforming growth factor-beta (TGF- β) and platelet-derived growth factor (PDGF), mediating HSC activation and fibrogenesis in zebrafish ALD models (Zhang *et al.*, 2017).

1.11.7 THERAPEUTIC INTERVENTIONS AND DRUG SCREENING

The adult zebrafish model offers a platform for screening potential therapeutic agents for ALD treatment. High-throughput drug screening assays using ethanol-

exposed zebrafish have identified compounds with hepatoprotective and anti-inflammatory properties. For example, natural compounds such as silymarin and curcumin have demonstrated protective effects against ethanol-induced liver injury in zebrafish, attenuating hepatocyte apoptosis and inflammation (Lin *et al.*, 2018; Lu *et al.*, 2016). Additionally, small molecule inhibitors targeting specific pathways implicated in ALD pathogenesis have shown promise in preclinical studies using zebrafish models (Choi *et al.*, 2018). The adult zebrafish model offers a valuable tool for investigating the pathogenesis of ALD and evaluating potential therapeutic interventions. With its amenability to genetic manipulation, optical imaging, and high-throughput drug screening, zebrafish complement existing animal models and provide unique insights into the complex mechanisms underlying ALD progression. Continued research using zebrafish models holds the promise of identifying novel therapeutic targets and advancing our understanding of ALD pathophysiology.

1.12 MONACOLIN X

Monacolin X is a natural compound derived from a marine sponge-associated fungal metabolite. It has been studied for its potential pharmacological properties, particularly its effects on angiogenesis and VEGFR2 signaling. Angiogenesis is the process of forming new blood vessels from pre-existing ones and is crucial for various physiological processes, including wound healing and development, but it can also be dysregulated in diseases like cancer, where excessive angiogenesis supports tumor growth and metastasis (Nagabhishek, S.N. *et al.*, 2019).

The marine sponge *Clathria frondifera* was collected from newline Gulf of Mannar and the crude extracts from the fungal strain NMKF7 and the newline bacterial strain NMKB17 were found to inhibit tube formation in HUVEC s newline cells. The strains were identified as *Monascus ruber* NMK7 using 18S rRNA newline for fungi and *Bacillus subtilis* NMK17 by 16S rRNA for bacteria. The extracts newline were subjected to column, TLC and HPLC chromatographic purification and newline screened all fractions and elutions through MTT to identify the active fractions newline PPCEF (partially purified crude extract Fungal) and PPCEB (partially purified newline crude extract bacteria). PPCEF and PPCEB has showed apoptotic property and newline antiangiogenic activity in breast cancer and endothelial cells. The presence of newline active compound from *Monascus*

rubin NMK7 was confirmed using Phospho-newline VEGFR-2 assay. HPLC purification and structural characterization using UV, newline IR, MS and NMR found out the active molecule Monacolin X with molecular newline weight of 418.5 Da and the formula C₂₄H₃₄O₆. Newline Monacolin X was found to induce dose-dependent cytotoxicity in newline different human breast cancer cell lines MCF-7, T47D, MDA-MB-231, MDAMB- newline468 and MCF-10A normal breast cell. Initially Monacolin X apoptotic newline activity was confirmed by PI and AO/EB staining in T47D and MDA-MB-468 newlineviii newlinecells. It has also induced the ROS stress (DCFH-DA), which causes the newline mitochondrial membrane potential difference (Rhodamine-123), the loss of cell newline membrane integrity, endoplasmic reticulum stress and eventually cell death. The newline initiation of apoptosis was proved by the increased expressions of Bax, cytochrome newlineC, cleaved PARP and cleaved caspase 3 followed by decreased expression of Bcl2 newline which were released due to the ER stress was confirmed by CHOP and ATF4 in in newline vitro breast cancer cell lines MDA-MB-468 and T47D studies (Nagabhishek, S.N. *et al.*, 2019).

1.12.1 CHEMICAL STRUCTURE

Monacolin X is structurally identical to lovastatin. It belongs to a class of compounds known as statins, which are inhibitors of the enzyme HMG-CoA reductase. This enzyme plays a crucial role in the synthesis of cholesterol in the liver.

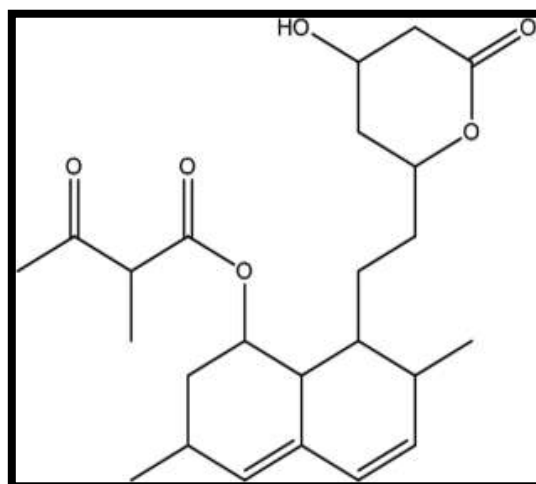


Fig:1.13 : Structure of Monacolin X

1.12.2 CHOLESTEROL LOWERING EFFECT

Monacolin X works by inhibiting HMG-CoA reductase, which reduces the production of cholesterol in the liver. By lowering cholesterol levels, it helps reduce the risk of cardiovascular diseases, including heart attacks and strokes.

Apart from inhibiting cell proliferation and migration, Monacolin X also demonstrates the ability to mitigate apoptosis induced by reactive oxygen species (ROS) stress, which is a significant factor in cancer progression. This suggests that Monacolin X could be a potential therapeutic agent for breast cancer treatment due to its multifaceted anti-cancer effects.

CHAPTER 2

LITERATURE SURVEY

2.1 LIVER DISEASE

Two million deaths per year are caused by liver disease, which also accounts for 4% of all deaths globally (1 out of every 25 deaths); men account for about two thirds of liver-related mortality (Asrani, S.K. *et al.*, 2019). This version of the global burden of liver disease is an update from 2019, with a primary focus on categories such as alcohol-associated liver disease, non-alcoholic fatty liver disease, viral hepatitis, and hepatocellular carcinoma (Mokdad, A.H. *et al.*, 2016). In this (Harshad Devarbhavi, *et al.*, 2023) they also include a distinct section on the prevalence of liver disease in Africa, a region of the world that is often overlooked in these kinds of publications (Devarbhavi, H. *et al.*, 2023). A major obstacle in treating malnutrition in infants and adults with advanced liver disease is the complex nature of the nutritional issues. This editorial offers commentary on the papers published in the "Nutrition and Liver Disease" special edition of *Nutrients*. The articles address a variety of diagnostic and treatment aspects that link the consequences of liver illness to dietary choices. Patients with advanced liver disease should have their nutritional condition evaluated as soon as possible and receive the necessary dietary therapies to sustain their nutritional status and prevent nutrition-related medical consequences. These patients' complex nutritional issues frequently originate from decreased intake brought on by anorexia, hypermetabolism, increased energy loss, and increased energy requirements. Reduced glycogen levels, decreased protein synthesis, decreased branched-chain amino acid (BCAA)/aromatic amino acid or BCCA/tyrosine ratios, and disruptions in fat metabolism during fasting are the hallmarks of the complex underlying metabolic situation has been reported (Mandato. C *et al.*, 2017). About 300 million individuals in China mainly suffer from liver illnesses, mostly from viral hepatitis (mainly from the Hepatitis B virus [HBV]), Nonalcoholic fatty liver disease, and Alcoholic liver disease was stated. The number of newly infected patients with HBV has significantly decreased since the expanded programme on Immunisation was established in 1992; yet, the number of patients with alcoholic and nonalcoholic fatty liver diseases is rising at an alarming rate was described. The

sharp rise in the quantity of papers in Hepatology indicates their intense activity in the field of liver disease research (Wang, F. *et al.*, 2014). In this review, they discussed the epidemiology and characteristics of liver diseases and liver-related research in China.

2.2 ALCOHOLIC LIVER DISEASE

Alcohol consumption and excessive drinking are the causes of alcoholic liver disease (ALD). Alcohol Dehydrogenases (ADHs) was a class of enzymes that oxidise organic chemical compounds to acetaldehyde after consumption of ethanol. The metabolic pathway that converts ethanol to acetaldehyde and then oxidises that acetaldehyde to acetate was largely carried out by the liver, and it is kinetically slower than other biological processes was described (Herbert Tilg *et al.*, 2011). Acetaldehyde was considered a carcinogen and was harmful to liver function, frequently leading to ALD. ALD may result in the formation of adducts, which are chemical alterations that may possibly interfere with normal biological functions. Reactive oxygen species (ROS) were also formed as a consequence of oxidising acetaldehyde to acetate. An overabundance of these molecules led to oxidative stress, which has been shown to play a critical role in Alzheimer's disease (ALD). From an immunological perspective, ALD was characterised by inflammation, involving both local and recruited inflammatory cells (Gyongyi Szabo *et al.*, 2010). When they were improperly activated, Kupffer cells (KCs), which are resident inflammatory cells in the liver, were found to be important in the onset of ALD. An increase in apoptosis-related cell death was also linked to ALD. This apoptotic cell death was thought to be caused by the elevated oxidative stress previously discussed and involved caspases as a method of execution (Li J., Zhao Y.R *et al.*, 2019).

The intricate immune responses involved in various liver illnesses and explained how a breakdown in liver homeostasis resulted in the progressive development of fibrotic tissue were reported by various studies (Bataller, R. and Brenner, D.A. 2005). They talked about a number of diseases that could cause liver cirrhosis, including chronic hepatitis B virus (HBV) and alcoholic liver disease. Because of their potential for tissue engineering and replacement, mesenchymal stem cells have been extensively investigated. The ability of MSCs to control the immune

system and change the course of a disease was covered in this article. Preclinical animal research was carried out with great effort, and the results that were obtained after host immunity inhibition were encouraging. The present status of mesenchymal stem cell clinical trials and other cellular and non-cellular therapeutics for liver cirrhosis diagnosis and therapy was finally summarised that in an attempt to replace injured hepatocytes, MSCs' differentiation capacity was harnessed. Crucially, MSCs exhibited immunomodulatory properties by encouraging T-reg activation and preventing the growth of cell types like macrophages and NK cells through cell-to-cell interaction and cytokine release. They have been proven to decrease the fibrotic development of cirrhosis and to deactivate hepatic stellate cells in mouse models. Numerous therapeutic approaches were investigated, including scaffold building, monoclonal antibodies, gene delivery and cell modification, molecular pathway targeting small medicines, and autologous MSC transplants has been described (Sarah Y. Neshat *et al.*, 2021).

There have been reports of dysbiosis and small intestine bacterial overgrowth developing in the context of ALD (Bode, Ch. *et al.*, 1990). Several explanations for these alterations have been proposed, such as altered host immunity and decreased intestinal motility leading to SIBO. Elevated levels of plasma endotoxins have been linked to ALD in both animal models and alcoholic people (Mathurin, P. 2000). Increased endotoxemia correlated with increased intestinal permeability. This was due to disruption of the gut barrier in ALD models. Mutlu *et al.* observed lower levels of Bacteroidetes and higher levels of Proteobacteria in a subgroup of alcoholic patients with and without liver impairment (Mutlu, E.A, *et al.*, 2012). Based on studies conducted on animals, it was proposed that the alterations in intestine microbial composition caused by alcohol could have a lasting effect. Additionally, ethanol may break down intercellular tight connections, which could lead to bacterial translocation. Hepatic receptors like TLR were activated by the passage of endotoxins or peptidoglycans, which led in the release of cytokines and the eventual deposition of fat and inflammation in the liver. Endogenous ethanol generated by bacteria exacerbated intestinal permeability and ultimately microbial translocation.

Adachi Y et al., 1995, reported that the therapies that target the gut microbiota were thought to be helpful for ALD. Based on research on animals, it was discovered that probiotics and antibiotics alleviated alcohol-induced liver damage and decreased endotoxemia (Loguercio C et al., 2005). Probiotics have been shown in certain trials to lower transaminase and cytokine levels in ALD. Similar to this, it has been demonstrated that rifaximin increases survival in alcoholic cirrhosis by altering the gut microbiota (Philips CA et al., 2018). Additionally, faecal microbial transplantation has been suggested to be helpful in the management of severe alcoholic hepatitis.

2.3 MONACOLIN X

(Sirpu Natesh Nagabhishek et al., 2019) For the first time, models of *in vivo*, *ex vivo*, *in vitro*, and *in silico* were used to investigate the mechanism and mode of action of Monacolin X, which is a strong inhibitor of angiogenesis and tumour angiogenesis that functions by downregulating the VEGFR2 pathway in response to VEGF stimulation. Furthermore, CAM assay, aortic ring assay, SIV and ISV development in zebrafish, and DMBA-induced breast cancer models demonstrated suppression of *in vivo* angiogenesis. It was also demonstrated to be a strong inhibitor in simulation experiments. Consequently, it was established that VEGFR2/Fik1/KDR was a viable target with great potential for additional lead optimisation as a chemotherapeutic drug (Nagabhishek, S.N et al., 2020).

Using the Phospho-newlineVEGFR-2 test, the existence of the newline active molecule from Monascus Ruber NMK7 was confirmed. Monacolin X, the active molecule, was identified through HPLC purification and structural characterisation using UV, newlineIR, MS, and NMR. Its molecular newline weight was 418.5 Da, and its formula was C₂₄H₃₄O₆. In different human breast cancer cell lines MCF-7, T47D, MDA-MB-231, MDA MB-newline468 and MCF-10A normal breast cell, newlinemonacolin X was found to elicit dose-dependent cytotoxicity. In T47D and MDA-MB-468 newlineviii newlinecells, PI and AO/EB staining were used to first validate the apoptotic newline activity of Monacolin X. Additionally, it created the ROS stress (DCFH-DA), which in turn led to the endoplasmic reticulum stress, the loss of cell integrity, the newline mitochondrial membrane potential difference (Rhodamine-123), and ultimately cell death. Increased expressions of Bax,

cytochrome newline C, cleaved PARP, and cleaved caspase 3 demonstrated the newlineinitiation of apoptosis, while decreased expression of Bcl2 newline, which was released as a result of ER stress, was verified by CHOP and ATF4 in *in vitro* studies using the breast cancer cell lines MDA-MB-468 and T47D.

The induction of nuclear damage and apoptosis by Monacolin X provides more evidence for the involvement of reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\psi_m$) (Nagabhishek, S.N. and Madankumar, A. 2019). Reactive oxygen species (ROS) can be advantageous or detrimental, depending on how much of them accumulate. ROS typically caused necrosis or apoptosis in cells, surpassing the capacity of the body's antioxidant defence systems. Additionally, mitochondria were essential for controlling other cellular processes like cell division and survival. Therefore, the mitochondrial membrane potential (MMP) was investigated using Rhodamine-123 to test for the involvement of mitochondria on treatment with Monacolin X induced cell death. An increase in green fluorescence in the treatment group indicated a possible difference in the mitochondrial membrane. Based on experimental data, it was determined that ROS may have mediated the nuclear damage that resulted in apoptosis by severely damaging the mitochondrial membrane. Using MDA-MB-468 and T47D cells, the scratch assay was used to test Monacolin X's anti-migratory ability. The results indicated that the compound had an anti-migratory effect when compared to the standard. In addition, the MIC technique verified that Monacolin X had outstanding antibacterial activity against both Gram-positive and negative pathogens. This suggested that the biological action of Monacolin X was promising (Nagabhishek, S.N. and Madankumar, A. 2019).

2.4 ZEBRAFISH (*Danio rerio*) AS A MODEL IN ETHANOL EXPOSURE

The hepatic steatosis and damage caused by ethanol in adult zebrafish were shown to be sufficiently produced by low dose chronic ethanol therapy (Ki-Hoon Park *et al.*, 2019). Researchers used treatment settings with 0.2% ethanol, which was similar to the blood ethanol levels seen in regular drinkers. As a result, they saw a marked rise in hepatic damage, as seen by the inflated hepatocyte accumulation and an elevation in blood ALT. Long-term ethanol exposure caused liver fibrosis in adult zebrafish, according to a prior study that used 1% ethanol

exposure settings. After 4 weeks of 0.2% ethanol therapy, we did see early stage moderate fibrosis in the liver, though this was not present in every group that was exposed to ethanol. In comparison to the prior work, they assumed that it was because of the comparatively shorter duration of ethanol exposure. However, considering that adult zebrafish hardly ate under 1% ethanol exposure circumstances, malnutrition may have had an impact on the fibrotic phenotype in the 1% ethanol exposure scenario. Researchers discovered that during the ethanol treatment phase, exposure to 0.2% ethanol did not impact hunger, suggesting that our model may have been more applicable to the chronic ALD disease. They also explored the possibility that hyper glycemia and hyper lipidemia, which are common in ALD patients and cannot be treated in a zebrafish larvae model, could be mimicked by low dosage chronic ethanol administration. Serum glucose, triacylglycerides, and ALT levels significantly increased in the blood sample analysis following ethanol administration. Their adult zebrafish model accurately replicated the metabolic and pathological alterations associated with ALD in the context of low-dose ethanol exposure (Park, K.-H. and Kim, S.-H. 2019).

The study was shown that long-term alcohol use decreased the intestinal absorption of riboflavin (Park, K.-H. and Kim, S.-H. 2020). Since riboflavin served as the building block for the synthesis of flavin adenine dinucleotide (FAD), ethanol consumption may cause the liver's levels of FAD to decrease. Since FAD was a necessary cofactor for many mitochondrial enzymes involved in protein folding, redox equilibrium, and energy metabolism, FAD reduction may have contributed to the liver damage caused by ethanol. When riboflavin supplementation was examined to see if it may lessen ethanol-induced hepatic injury, it was discovered to inhibit ethanol-induced hepatic injury. Additionally, it was discovered that adult zebrafish treated with riboflavin or NR showed dose-dependent reduction of hepatic damage when exposed to ethanol.

Oil red staining showed that after four weeks of exposure to 0.5% ethanol, zebrafish developed severe liver steatosis (Schneider, A.C.R. *et al.*, 2017). The primary ultrastructural alterations in the ethanol-treated mice were associated with an increase in rough endoplasmic reticulum cisterns, glycogen particles, and cytoplasmic lipid droplets. Hepatic mRNA expression of adipor2, sirt-1, and il-1b

was elevated between two and four weeks, suggesting that ethanol triggered signalling molecules that were important for both hepatic protective and inflammatory responses. Animals treated to ethanol did not exhibit any detectable levels of adiponectin in their livers, and there was no discernible variation in il-10 levels either. Research revealed that in zebrafish exposed to ethanol on a long-term basis, inflammatory signalling and ultrastructural changes were important factors in the development of hepatic steatosis (Jang, Z.-H. *et al.*, 2012).

The present investigation used a zebrafish disease model to undertake metabolic profile of an alcoholic fatty liver using gas chromatography-mass spectrometry (GC-MS) and proton nuclear magnetic resonance spectroscopy (¹H-NMR). To find out how metabolism was controlled in an alcoholic fatty liver, metabolic differences were analysed between the control and alcoholic fatty liver groups in zebrafish. There was a substantial difference between the control and alcoholic fatty liver groups, according to multivariate statistical analysis. Excretion of isoleucine, acetate, succinate, choline, creatine, acetoacetate, 3-hydroxybutyrate (3HB), ethyl glucuronide (EtG), lactate/pyruvate ratio, fatty acids, and cholesterol was increased in the alcoholic fatty liver group, while it was decreased in the group that excreted citrate, aspartate, tyrosine, glycine, glucose, alanine, betaine, and maltose. Long-term alcohol use has been related to metabolites found in the fatty liver groups, which resulted in oxidative stress and oxidation-reduction (redox) alterations. According to this study, zebrafish model global metabolite profiling may shed light on the metabolic alterations in alcoholic fatty livers.

CHAPTER 3

AIM AND SCOPE

3.1 AIM

This present study was investigated the hepato-protective effect of Monacolin X, which can mitigate through the adverse effects of chronic ethanol mediated liver disease. We focused on assessing changes in hepatic steatosis and evaluating markers of liver injury in presence of Monacolin X.

3.2 SCOPE

- ✚ To evaluate the protective effects of Monacolin X on chronic ethanol-induced hepatic steatosis, focusing on pathological changes within the liver. Chronic hepatic steatosis, characterized by excessive fat accumulation in the liver, is a common precursor to more severe liver diseases such as alcoholic liver disease (ALD). Ethanol consumption is a major risk factor for the development of hepatic steatosis and subsequent liver injury has been reported.
- ✚ Monacolin X is a natural compound derived from a marine sponge-associated fungal metabolite. It has shown promising hepatoprotective properties in preclinical studies. This research seeks to elucidate whether Monacolin X can mitigate ethanol-induced hepatic steatosis and injury through its potential anti-inflammatory, antioxidant, and lipid-lowering mechanisms.
- ✚ To determine how the drug mitigate the effect of alcohol and prevents hepatic steatosis.
- ✚ Findings from this research could contribute to our understanding of novel therapeutic strategies for preventing and managing alcoholic liver disease, offering insights into the efficacy and mechanisms of action of Monacolin X in protecting against ethanol-induced liver damage.
- ✚ This study has involved animal models exposed to chronic ethanol consumption, with some subjects receiving Monacolin X supplementation while others serve as controls. Various parameters including liver histology, biochemical markers of liver function, and inflammatory markers will be

assessed to evaluate the extent of hepatic steatosis and injury, as well as the potential protective effects of Monacolin X.

- ✚ Parameters involves to quantify the total protein content and liver markers Serum Glutamic Oxaloacetic Transaminase (SGOT) and Serum Glutamate Pyruvate Transaminase(SGPT) levels.
- ✚ To quantify the level of oxidative stress. Superoxide Dismutase(SOD), Catalase (CAT), Glutathione Peroxidase (GPx), Glutathione S-Transferase (GST) and Glutathione (GSH).
- ✚ To quantify gene expressions of inflammatory markers (TNF α , NFkB, IL-1 β and IL6).

CHAPTER 4

MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMAL MODEL

Adult zebrafish (*Danio rerio*) has chosen as an experimental animal model system to carry out the hepatoprotective activity of Monacolin X against alcohol mediated acute and chronic liver injury. This study was approved by the Institutional Animal Ethics Committee (IAEC) granted its approval number **(SU/CLATR/IAEC/XXII/29/2024)** through The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

4.1.1 ZEBRAFISH MAINTENANCE

The experimental procedures was used in this study was carried out in accordance with applicable regulations (Ki-Hoon Park *et al.*, 2020). A few gallons of water in the glass fish tanks that constantly run with air pump was used to keep zebrafish in an aquatic environment that is favourable to health and development of the fish. Generally, the tank or room temperature is kept at a normal level. Freshwater zebrafish (*Danio rerio*) was kept with 12 h/12 h light and dark cycle in a glass tank. The glass aquarium (60 cm × 30 cm × 25 cm) was maintained with dechlorinated water. About 10 fishes were maintained for each set of experiment and acclimatized prior to the experiment at 28°C ± 2°C.

4.1.2 ZEBRAFISH SPECIES

The zebrafish strains used in this study was wild type (5months old). Both Male & Female gender has been chosen randomly. Siblings of the wild type zebrafish's were fed normal fish food (Optimum, Highly Nutritious micro pellet food, rich in vitamins C and E, purchased from southern India Aqua Tek, Chennai) once a day.

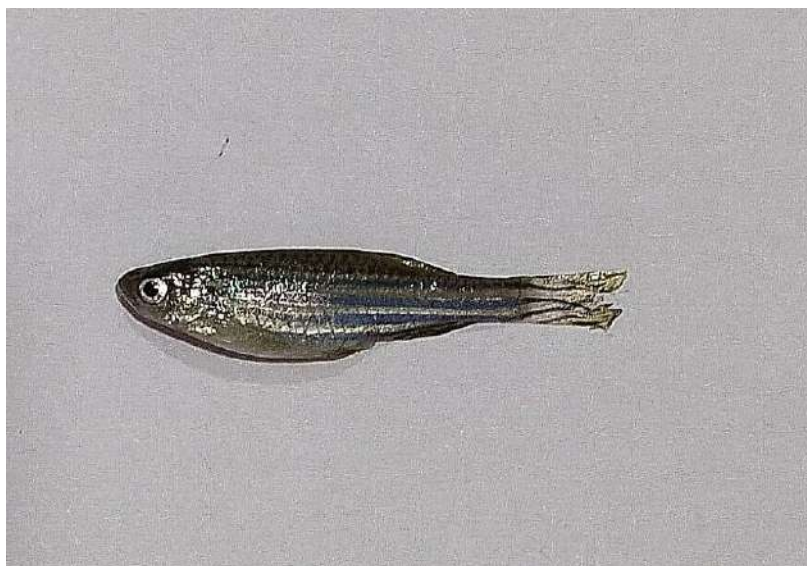


Fig:4.1 : ZEBRAFISH (*Danio rerio*)

4.2 EXPERIMENTAL DESIGN

4.2.1 ZEBRAFISH GROUPS

Groups of wild type siblings (10 fishes per tank) were randomly selected and maintained in separated 3-gallon glass tanks containing 1L of water. Water was exchanged once every 24 hours. Gallon glass tank (10 fishes) were separated as Control, Ethanol induction, Ethanol + Monacolin X.

4.2.2 CONTROL

In one gallon glass tank (10 fishes) were separated for control group. Water was exchanged once every 24 hours. The fishes were fed twice a day during experimental period.

4.2.3 ETHANOL TREATMENT

In one gallon glass tank (10 fishes) were induced with 0.5% Ethanol (v/v) in 1L of water. Ethanol containing water was exchanged once every 24 hours. An isolated glass tank were protected with glass lids to reduce the amount of ethanol evaporation, which is influenced by fish metabolism and evaporation (Park, K.-H. and Kim, S.-H. 2019).

4.2.4 MONACOLIN X TREATMENT

In another gallon of glass tank (10 fishes) were induced with 0.5% Ethanol with 2 μ M Monacolin X. Monacolin X (Stock Concentration=10mg/ml) 1mg was dissolved in 1ml of ethanol. Monacolin X was dissolved in the respective experimental water tank to a group of zebrafish by swallowing.

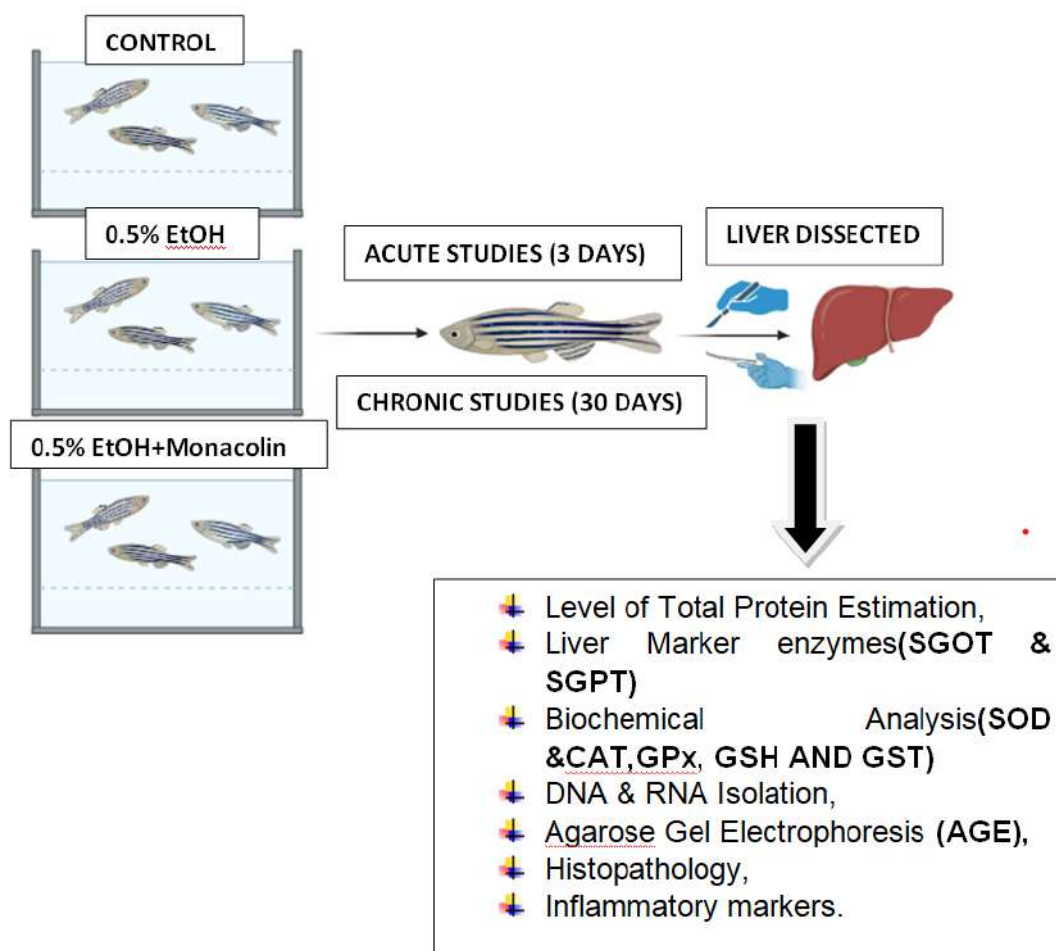


Fig:4.2 : Experimental Design of Acute and Chronic studies with illustration of experiments

4.3 EXPERIMENTAL DURATION

4.3.1 ACUTE STUDIES

Three groups were separated (Control, 0.5% Ethanol, 0.5% Ethanol + Monacolin X) respectively contains 10 fishes of each groups. The duration period of acute studies were 3 days induction. Adult zebrafish were exposed for 24, 48, and 72

hours. The fish were fed twice a day and the water was changed constantly each 24 hours.

4.3.2 CHRONIC STUDIES

Three groups were separated as same as acute studies (Control, 0.5% Ethanol, 0.5% Ethanol + Monacolin X) respectively containing 10 fish each. The duration of chronic studies was a 30 days induction. The fish were fed twice a day and the water was changed constantly each 24 hours as in acute study.

4.4 BLOOD COLLECTION AND DISSECTION

4.4.1 MATERIALS

- ✚ Insulin syringe,
- ✚ EDTA,
- ✚ 1X Phosphate Buffer,
- ✚ Ice pack,
- ✚ 1.5ml Eppendorf tubes,
- ✚ Centrifuge

4.4.2 BLOOD COLLECTION METHOD

Prepare the materials needed for blood collection. The blood samples were collected after 72 hours of induction. In the sterile environment the fish were taken from tank, placed on ice pack for few seconds and using insulin syringe, EDTA was punctured & blood was collected in the area of the dorsal aorta, posterior to the anus and along the body axis. Keep the syringe tip away from any potential contamination sources. An average of between 5 to 20 μ L of blood was extracted from a fish. The size of the fish determines how much blood can be extracted. Samples of blood were diluted 1:10 in PBS. It's also essential to secure the blood sample in the centrifuge within ten minutes of blood collection to prevent hemolysis (Gabriela L Pedroso *et al.*, 2012). Centrifuge the blood at 700g for 3 mins, serum supernatant was collected. The serum was at the tube's top layer after centrifugation. The serum was aspirated using a pipette to ensure that only the essential serum was obtained, keeping the layers stable and clearly separated. After that, the serum was put into a fresh microtube and prepared for use in

biochemical analyses. While awaiting the start of the biochemical analyses, it was kept on ice.



Fig:4.3 : Blood collection from zebrafish (Danio rerio)

4.4.3 MATERIALS:

- ✚ Ice pack,
- ✚ Paper towel,
- ✚ Dissection Board,
- ✚ Dissection Blade,
- ✚ Eppendorf Tube,
- ✚ 1X Phosphate Buffer,
- ✚ 10% Formalin,
- ✚ 70% Ethanol.

4.4.4 DISSECTION:

Primarily, incubation in ice pack for 5 minutes is required before dissecting. First, use a paper towel to gently pat the fish, dry before placing it on a dissection board. Zebrafish have paired pectoral and pelvic fins and a single dorsal, caudal, and anal fin on their exterior. The skin on the belly of the fish was snipped just anterior to the anal fin. From the anal fin to the operculum, the skin and underlying muscle were severed along the abdomen (the hard covering over the gill). The operculum and pectoral fin were then extracted, together with the pectoral girdle, which is the thick, bony area at the base of the fin. From above the now-exposed gill posteriorly along the fish's side, down to the anal fin, the skin and underlying muscle were severed.

Many of the fish's internal organs were visible once the skin and underlying muscle were gently peeled from the side. The fish's gastrointestinal tract was taken out of

its body. Large size, lobed morphology, tannish colour, and abundant vascularization are characteristics that help identify the liver. The dissected liver was placed in fresh eppendorf tube with 1X PBS. The remaining whole body tissue were placed in eppendorf tube with 1X PBS immersion (Gupta, T. and Mullins, M.C. 2010). Same procedure were followed to ethanol mediated chronic liver injury study.



Fig:4.4 : Dissection of zebrafish (*Danio rerio*)

4.4.4.1 DISSECTION GROUPS

The set of each groups (Control, 0.5% Ethanol, 0.5% Ethanol + Monacolin X) were dissected for three various analysis. The Dissection for biochemical estimation includes blood collection, liver collection and whole body tissue immersed with 1X PBS. The RNA isolation was done by taking few liver samples and put in trizol reagent by a set from each groups were dissected by cutting the head & tail region of the fish. The liver was taken off and immersed with trizol reagents for gene expression. For Histology, the fish's tail was removed and stored in 10% buffered formalin. After 24 hours of formalin, the solution were freshly made and changed. After 48 hours formalin solution was discarded and 70% Ethanol was immersed (Moore, J.L. *et al.*, 2002). Transfer the samples to closed container, the lid was tightened, and the container lid interface was covered with Parafilm. The remaining tissues were stored, in -80C immediately. The samples were submitted to histopathology lab. (Sathyabama Dental College and Hospital, Chennai, India).

4.5 SAMPLE PREPARATION

4.5.1 MATERIALS

- ✚ Eppendorf tube,
- ✚ 1X Phosphate Buffer (PBS),
- ✚ Sonicator,
- ✚ Centrifuge

The dissected samples were transferred to a suitable homogenization vessel depending on the volume. We transferred to the eppendorf tube immersed with 1X PBS. The sonicator was operated at the proper settings for the required time and intensity of homogenization (Required time 20 minutes). Sonication was applied to the zebrafish samples, where the sonicator probe or horn was submerged in the sample and turned on. The samples were examined once the homogenization procedure was finished to make sure that the desired level of homogenization had been reached. After being homogenised, the zebrafish samples were prepared for additional analysis. The homogenised samples were centrifuged at 1000rpm for 3 minutes. Supernatant was collected. Further the samples were diluted at 1:10 ratio.

4.6 LEVEL OF TOTAL PROTEIN ESTIMATION

4.6.1 MATERIALS

- ✚ Standard Bovine Serum Albumin (BSA) (Concentration=100µg/ml),
- ✚ Alkaline copper sulphate reagent,
- ✚ Folin's reagent (Dilution=1:2),
- ✚ Distilled water,
- ✚ Spectrophotometer.

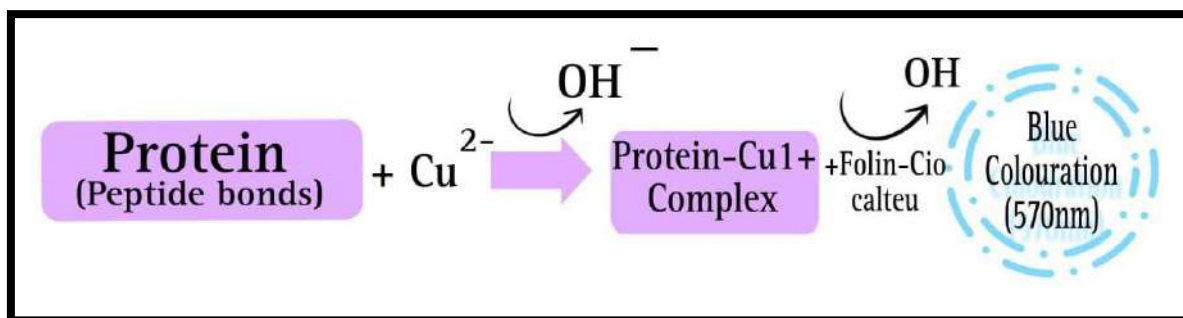


Fig:4.5 : Reaction of protein estimation.

4.6.2 PROCEDURE

The serum was collected from blood samples of different groups such as Control, 0.5% of Ethanol and 0.5% of Ethanol + Monacolin X for both acute and chronic species. The serum, liver and whole body tissue samples were diluted at 1:10 ratio. A series of BSA standard solutions with known concentrations were prepared (2mg/ml conc.). An Alkaline copper sulphate solution, Folin-Ciocalteu reagent at a ratio of 1:1 was prepared. Standard solution and protein sample were pipetted into separate wells of a 96 well plate. Following distilled water was added to each well and alkaline copper sulphate was added to each well. After incubation of 10 minutes, Folin's reagent was added. Gently mixed by tapping or swirling. The plate were incubated at room temperature for 30 minutes to allow colour development. After incubation, the absorbance (OD value) of each well was measured at 570 nm using a spectrophotometer (Waterborg, J.H. and Matthews, H.R. 1984).

4.6.3 LEVEL OF TOTAL PROTEIN CONCENTRATION

The level of total protein concentration was calculated using the formula given below

$$\frac{(\text{Test OD})}{0.06} \times \text{sample vol} \times \frac{10}{0.1} \times \frac{1}{1000} \text{ mg of protein}$$

4.7 LIVER MARKER ENZYMES ANALYSIS

Serum Glutamic Oxaloacetic Transaminase (SGOT) and Serum Glutamic Pyruvate Transaminase (SGPT), also known as Aspartate Amino Transferase (AST) and Alanine Amino Transferase (ALT) respectively, are enzymes primarily

found in the liver but also in other tissues such as the heart and muscles. They play a crucial role in amino acid metabolism. In ethanol-induced liver damage, these enzymes are often used as markers to assess the extent of liver injury, as their levels can increase when liver cells are damaged or destroyed.

4.7.1 MATERIALS

- ✚ Buffer Substrate (Aspartic Acid & Alanine),
- ✚ Dinitrophenyl Hydrazine (DNPH),
- ✚ 0.4N NaOH.

4.7.2 PROCEDURE

The buffer substrate (Aspartic Acid or SGOT, Alanine or SGPT) was added in 96 well plates each, by placing the plate on ice pack. The blood and liver samples were added accordingly (Triplicates) for both acute and chronic groups. Further DNPH was added and covered with foil paper. Incubate the plates at room temperature for 20 minutes. After incubation NaOH was pipetted. Gently mixed by tapping or swirling. After incubation, the absorbance (O.D value) of each well was measured at 540 nm using a spectrophotometer.

4.7.3 ACTIVITY OF SGOT & SGPT

The activity of SGOT & SGPT was calculated using the formula given below

$$\frac{\text{Test OD}}{0.01} \times 10 \times \frac{1}{60} \text{ is normalised to mg of protein}$$

4.8 BIOCHEMICAL ANALYSIS (OXIDATIVE STRESS MARKERS)

Biochemical analysis includes an antioxidant enzyme activity of Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxide (GPx), Glutathione (GSH) and Glutathione S-transferase (GST).

4.8.1 SUPEROXIDE DISMUTASE (SOD)

Superoxide dismutase is an important antioxidant enzyme that catalyzes the dismutation of superoxide radicals ($O_2^{\cdot -}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2), thus protecting cells from oxidative stress. The amount of oxidative stress that is present in liver samples can be inferred from measurements of SOD

activity. Increased production of ROS may be indicated by higher SOD activity, whereas lower activity may indicate impairment of antioxidant defence mechanisms.

4.8.1.1 MATERIALS

- ✚ Absolute ethanol,
- ✚ Chloroform,
- ✚ 0.1M Tris buffer(pH 8.2),
- ✚ Distilled water,
- ✚ Pyrogallol.

4.8.1.2 PROCEDURE

The blood and liver samples were added in 96 well plates each (Triplicates). Blank was included, followed by Ethanol, Chloroform was added. Incubated the wells for 15 minutes at room temperature. After incubation Tris, distilled water and pyrogallol was pipetted. The absorbance (OD value) of each well was measured at 470nm using spectrophotometer.

4.8.1.3 ACTIVITY OF SOD

The activity of SOD was calculated using the formula given

$$\frac{OD}{10} \times \frac{0.2}{0.01} \times \frac{1}{0.5} \text{ is normalised to mg of protein}$$

4.8.2 CATALASE (CAT)

Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide (H₂O₂) into water (H₂O) and oxygen (O₂). This measures the activity of catalase by monitoring the decomposition of hydrogen peroxide, often by observing the rate of oxygen evolution.

4.8.2.1 MATERIALS

- ✚ Phosphate buffer,
- ✚ 0.2M Hydrogen peroxide (H₂O₂),
- ✚ Potassium dichromate (K₂Cr₂O₇).

4.8.2.2 PROCEDURE

The blood, liver and whole body tissues samples were added in 96 well plates (Triplicates). Blank was included, phosphate buffer was added. After H₂O₂ and K₂Cr₂O₇ was pipette. Incubated the wells for 10 minutes. After incubation, the absorbance (OD value) of each well was measured at 590nm using spectrophotometer.

4.8.2.3 ACTIVITY OF CAT

The activity of CAT was calculated using the formula given below

$$\frac{\text{Test OD}}{\text{Blank OD}} \times 3.4 \times \frac{1000}{0.034} \times \frac{10}{0.1} \text{ is normalised to mg of protein}$$

4.8.3 GLUTATHIONE PEROXIDE (GPx)

Glutathione peroxidase is an enzyme that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides, using glutathione as a cofactor. This measures the ability of this enzyme to reduce peroxides, often by following the oxidation of reduced glutathione.

4.8.3.1 MATERIALS

- ✚ 0.04M Phosphate Buffer (PO₄),
- ✚ 10mM Sodium azide (NaN₃),
- ✚ 4mM Reduced Glutathione (GSH),
- ✚ 2.5mM Hydrogen peroxide (H₂O₂),
- ✚ 10% Trichloroacetic acid (TCA),
- ✚ Dithio nitrobenzoic acid (DTNB).

4.8.3.2 PROCEDURE

The samples of Liver and whole body tissues was added in 96 well plates (Triplicates) each. The Phosphate buffer was added, sodium azide, GSH, hydrogen peroxide, distilled water were added continuously. Incubated the wells for 3 minutes. After incubation DTNB and TCA were added. The absorbance (OD value) of each well was measured at 412nm using spectrophotometer.

4.8.3.3 ACTIVITY OF GPx

The activity of GPx was calculated using the formula given below

$$\frac{\text{Test OD}}{0.11} \times 10 \times \frac{2}{0.01} \times \frac{1}{0.2} \text{ is normalised to mg of protein}$$

4.8.4 GLUTATHIONE (GSH):

Glutathione is a tripeptide antioxidant that plays a key role in cellular defence against oxidative stress. It exists in both reduced (GSH) and oxidized (GSSG) forms. This typically measures the total glutathione content (GSH + GSSG) or specifically the reduced glutathione (GSH) content in a sample, often using enzymatic recycling assays or chromatographic methods.

4.8.4.1 MATERIALS

- ✚ 10% Trichloroacetic acid (TCA)
- ✚ 0.3M Phosphate buffer (PO4)
- ✚ Dithio nitrobenzoic acid (DTNB)

4.8.4.2 PROCEDURE

The samples of Liver and whole body tissues was added in 96 well plates (Triplicates) each. The 10% TCA was added, after that phosphate buffer was added. DTNB was added to every wells. Incubate for 10 minutes at room temperature. After incubation, the absorbance (OD value) of each well was measured at 412nm using spectrophotometer.

4.8.4.3 ACTIVITY OF GSH

The activity of GSH was calculated using the formula given below

$$\frac{\text{Test OD}}{0.11} \times 10 \times \frac{2}{0.01} \times \frac{1}{0.2} \text{ is normalised to mg of protein}$$

4.8.5 GLUTATHIONE S TRANSFERASE (GST)

Glutathione S-transferases are a family of enzymes that catalyze the conjugation of reduced glutathione (GSH) with electrophilic substrates, making them more water-soluble and easier to excrete. The GST assay measures the activity of these

enzymes, often by monitoring the formation of glutathione-substrate conjugates spectrophotometrically or by other methods.

4.8.5.1 MATERIALS

- ✚ 0.5M Phosphate Buffer (PBS) (ph 6.5),
- ✚ 25mM Chloro dinitro benzene (CDNB),
- ✚ Glutathione (GSH)

4.8.5.2 PROCEDURE

The samples of Liver and whole body tissues was added in 96 well plates (Triplicates) each. The CDNB was added, after phosphate buffer was added. Incubate for 5 minutes. After incubation, GSH was pipetted. The absorbance (OD value) of each well was measured at 340nm using spectrophotometer.

4.8.5.3 ACTIVITY OF GSH

The activity of GSH was calculated using the formula given below

$$\frac{\text{Test OD}}{0.11} \times 10 \times \frac{2}{0.01} \times \frac{1}{0.2} \text{ is normalised to mg of protein}$$

4.9 DNA ISOLATION

DNA isolation, which can also be referred to as DNA extraction or DNA purification, is the procedure used to separate DNA molecules from biological materials including tissues, cells, or organisms. Pursuing pure DNA for a range of subsequent uses was the aim of the DNA isolation process.

4.9.1 MATERIALS

- ✚ Homogenize Buffer,
- ✚ Sonicator,
- ✚ 20%SDS,
- ✚ Phenol,
- ✚ Chloroform
- ✚ Isoamyl Alcohol
- ✚ Centrifuge,
- ✚ 60% Ethanol

✚ Sterile water.

4.9.2 PROCEDURE

The Homogenize buffer was added to the whole body tissue samples for both acute and chronic samples. The samples were homogenized well using sonicator. After homogenized, centrifuge at 1000rpm for 5 minutes. The supernatant was collected. 20% SDS and Phenol was added to the samples. Chloroform & Isoamyl alcohol at 24:1 ratio was mixed with the samples. The tubes were shaken vigorously and centrifuge at 11,000rpm for 5 minutes. The organic (bottom) and aqueous (top) phases were separated. Moved the DNA-containing aqueous phase to a new tube, taking care not to disturb the organic or interphase phases in the process. 4M NaCl was added drop by drop. 100% ethanol was added and centrifuged at 12,000rpm for 5 minutes. Discard the supernatant, pellet was formed tends to wash with 60% ethanol twice. Air dried the tube and sterile water was added and stored at -20°C for further analysis.

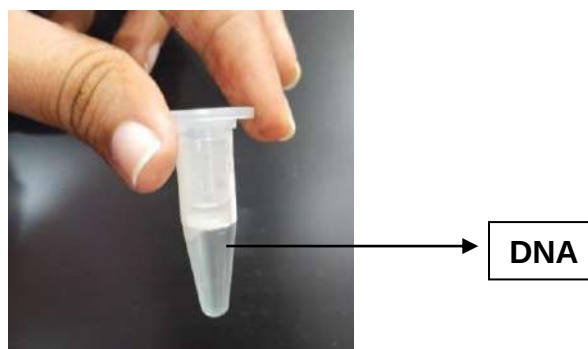


Fig:4.6 : ISOLATION OF DNA FROM LIVERS OF EXPERIMENTAL ZEBRAFISH

4.10 AGAROSE GEL ELECTROPHORESIS

The principle of agarose gel electrophoresis involves separating DNA molecules based on their size and charge. When an electric current is applied to the gel, negatively charged DNA fragments move towards the positively charged electrode. Smaller fragments move faster and travel farther through the gel matrix than larger ones, resulting in distinct bands that can be visualized using stains like ethidium bromide.

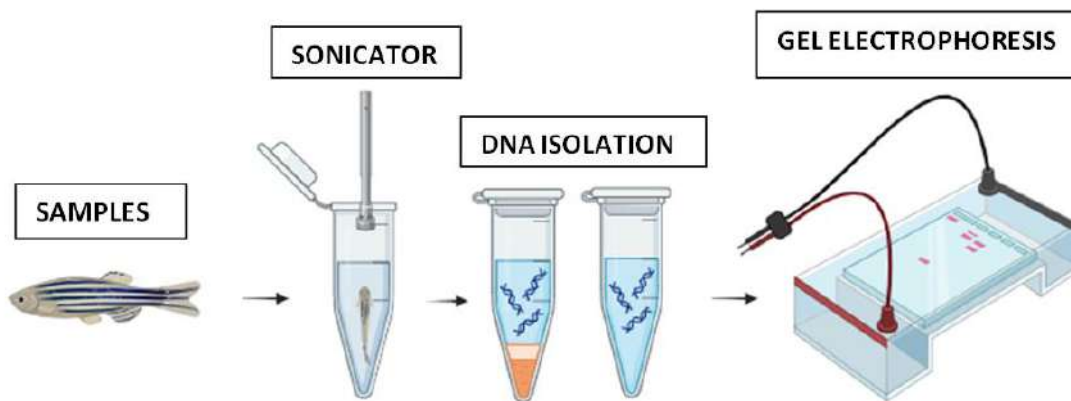


Fig:4.7 : Illustration of sample homogenised using sonicator, DNA isolation and Gel electrophoresis.

4.10.1 MATERIALS

- ✚ 1X TBE (Tris boric acid EDTA) buffer
- ✚ 1% Agarose
- ✚ EtBr (Ethidium Bromide)
- ✚ Casting tray and comb
- ✚ Electrophoresis unit tank
- ✚ Power supply
- ✚ Bromophenol blue
- ✚ Glycerol
- ✚ DNA samples

4.10.2 PROCEDURE

TBE buffer 10X was prepared by adding tris base, boric acid and EDTA into deionized water. Then the 10x was converted into 1x by diluting in deionized water. 1% agarose gel was made with TBE buffer. It was boiled using microwave oven, until the agarose dissolved properly. It was then cooled down to room temperature. Once cooled down EtBr was added. In the meantime, the gel casting tray was made ready by tapping the ends securely and the comb was placed. Then it was poured into a gel casting tray with the comb. It was allowed to solidify. Once solidified the comb was removed gently. The tray was placed in the electrophoresis unit tank and 1x TBE buffer was poured till the gel was submerged. Gel-loading dye was prepared using glycerol, 1x TBE buffer and bromophenol blue. To each well DNA sample and gel loading dye were mixed and

loaded. It was connected to the power supply and was set at 100V. Once the DNA ran up to the desired level, the power supply was turned off. Then the gel was visualized under UV light using Chemidoc Instrument.

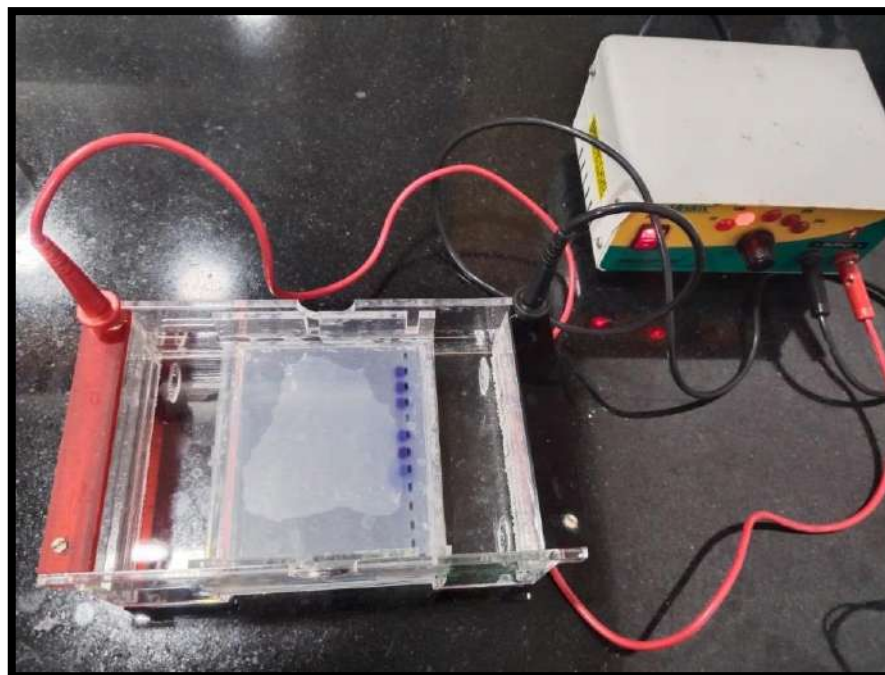


Fig:4.8 : Processing of Agarose Gel Electrophoresis

4.11 RNA ISOLATION

The concept behind RNA isolation is to remove specific RNA molecules from a biological sample while limiting contamination and maintaining the integrity of the molecules. Generally, the mechanism takes advantage of the distinctions in chemical characteristics between RNA and other constituents of the cell, like proteins, lipids, and DNA.

4.11.1 MATERIALS

- ✚ Trizol,
- ✚ Chloroform,
- ✚ Isopropanol,
- ✚ 95% Ethanol,
- ✚ RNase-free water.

4.11.2 PROCEDURE

The trizol stored samples were homogenized in ice cold condition. Chilled chloroform was added and vortexed for 15 seconds to mix it thoroughly. Then incubated at room temp for 2-3 minutes and centrifuged at 8000 rpm for 15 minutes at 4°C. 3 phases were visible, and the aqueous layer was transferred to another tube. Ice-chilled isopropanol was added and incubated for 15 minutes at room temperature and it was centrifuged at 8000 rpm for 15 min at 4°C. The supernatant was drained off and the pellet was collected. The pellet was washed with 95% ethanol, it was prepared with RNase-free water. Then centrifuged at 3000 rpm for 15 minutes at 4°C. Ethanol was removed carefully and washed twice. After that, it was allowed to air dry in the hood but not completely. Once air-dried 80-90% RNase-free water was added and stored at -20°C.

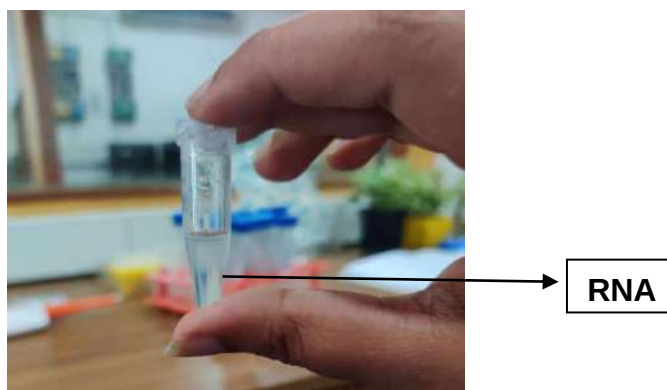


Fig:4.9 : ISOLATION OF RNA FROM LIVERS OF EXPERIMENTAL ZEBRAFISH

4.12 COMPLEMENTARY DNA (cDNA) CONVERSION

One of the most important processes in molecular biology is the conversion of RNA into cDNA, which allows for the use of cDNA in cloning and other downstream applications like gene expression research. To guarantee the integrity and calibre of the RNA template, this procedure usually starts with the isolation and purification of RNA from the intended biological sample. By attaching themselves to the poly(A) tail of mRNA molecules, oligo (dT) primers are frequently used to start the reverse transcription step. Reverse transcriptase uses this hybrid primer-template to begin the process of synthesizing single-stranded cDNA by employing the RNA template. The reverse transcriptase enzyme is able to catalyze the conversion of RNA into cDNA when the reverse transcription

reaction is conducted under carefully regulated circumstances, typically at a temperature of 37 to 42°C. The reaction is stopped after the proper incubation time, usually by heating the enzyme to inactivation. It is possible to guarantee the purity of the cDNA result by employing RNA-specific enzymes to eliminate any remaining RNA.

4.12.1 PROCEDURE

Using the iScript™ cDNA Synthesis Kit, Total RNA was transformed to cDNA as per the manufacturer's instructions. Following the technique, Total RNA, Master mix, and Reverse transcriptase (RT) enzyme were combined to create a standard volume. The tubes underwent an incubation period of five minutes at 25°C for priming, 46°C for RT enzyme activation, one minute at 95°C for reverse transcriptase inactivation, and then four minutes at 4°C. For further usage, the tubes were kept in storage at -20°C.

4.13 PRIMER DESIGNING

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

Table 4.1 : List of Primers used for qRT-PCR.

S.NO	TARGET GENE	GENE ID	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')	AMPLICON LENGTH (bp)	Ta (°C)
1.	NF-κB	4791	CGAATGGA CAAGACAG CAG	CCGGAACAC AATGGCATA	177	53.7
2.	IL-6	3569	ATAACCACC CCTGACCC CAAC	ATACCTCAA CTCCAAAAG ACC	149	55.1
3.	IL-1β	7124	CTCTCACCT CTCCTACTC AC	ACACTGCTA CTTCTTGCC C	194	54.3
4.	TNF-α	3553	AACCATTCT CCTTCTCCC C	TTTCACCTCC ATCCATCCT C	186	51.3

4.14 QUANTITATIVE REAL-TIME-POLYMERASE CHAIN REACTION (qRT-PCR)

Using quantitative real-time polymerase chain reaction (qRT-PCR), the gene expression levels of NF-κB, IL-6 and IL-1β and TNF-α and were examined. For each target gene, gene-specific primers were used in accordance with the guidelines supplied by the SensiFAST™ SYBR® Lo-ROX Kit from Bioline, UK. To assure accuracy, duplicate analyses were performed on each sample. SYBR Green master mix, Forward and reverse primers and cDNA template were combined for the qRT-PCR setup. The volume was then adjusted by using nuclease-free water. Using the Master cycler Nexus Gradient Thermal Cycler from Eppendorf, Germany, PCR amplification was performed under the following thermal cycling conditions: 2 minutes of initial polymerase activation at 95°C, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60 to 65°C for 10 sec, and extension at 72°C for 15 sec. For 40 cycles, the cycle threshold value (Ct value) has been determined to measure the levels of gene expression. The 2-

$\Delta\Delta C_t$ procedure was utilized for relative quantification, with the assumption that the reference sample had a relative gene expression of 1. The results were expressed as an expression fold change relative to the control, where a fold change of more than 1.0 indicated upregulation of the gene and less than 1.0 indicated downregulation. This thorough qRT-PCR study offers insightful information on the NF- κ B, IL6, IL1 β , and TNF α gene expression patterns under the experimental circumstances.

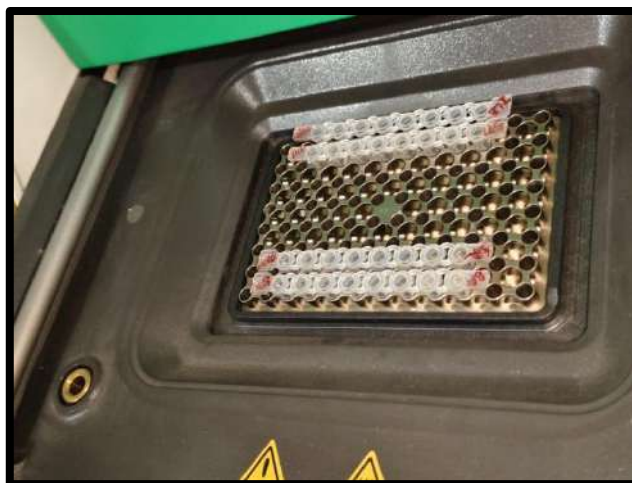


Fig:4.10 : qRT-PCR

4.15 HISTOPATHOLOGY

The histopathology of an inflamed zebrafish liver slice usually exhibits a number of distinctive characteristics. Hepatocytes may sustain damage from inflammation, which could result in changes to their morphology. The samples which are preserved with 10% Formalin were given for histopathology at Sathyabama Dental College. The received slides were observed under the Light Microscope for morphological changes in the liver.



Fig:4.11 : 10% Buffered formalin stored samples for Histopathology

4.15.1 HAEMATOXYLIN & EOSIN STAINING

Hematoxylin and Eosin (H&E) staining is a fundamental histological technique used to visualize tissue structure and morphology.

4.15.1.1 MATERIALS

- ✚ Xylene,
- ✚ Graded Alcohol solutions,
- ✚ Hematoxylin solution,
- ✚ Distilled water,
- ✚ Eosin,
- ✚ Mounting medium,
- ✚ Microscope slides & cover slips.

4.15.1.2 PROCEDURE

First, the liver tissue sections were deparaffinized and hydrated using xylene and graded alcohol solutions. This process removed the embedding medium and rehydrated the tissue, preparing it for staining (Nagata, K. *et al.*, 1989). Next, the tissue sections were immersed in Hematoxylin solution, which selectively stained cell nuclei blue-purple. Hematoxylin bound to acidic components of the nucleus, providing contrast and highlighting cellular morphology. After Hematoxylin staining, the sections were rinsed in water to remove excess stain. Subsequently, the

sections were counterstained with Eosin, a pink-red dye that bound to basic components of the tissue, such as cytoplasm and extracellular matrix. Eosin staining helped differentiate various cellular structures and enhanced contrast in the tissue. Following eosin staining, the sections were dehydrated using graded alcohol solutions to remove excess water and facilitate mounting (Sullivan-Brown, J. *et al.*, 2010). Finally, the sections were cleared in xylene and coverslipped using a mounting medium to preserve the stained tissue and facilitate microscopic examination. This H&E staining procedure allowed for the visualization of liver tissue architecture, including hepatocytes, sinusoids, bile ducts, and other structural elements.

4.15.2 MASSONS TRICHROME STAINING

Tissue samples were stained with Masson's trichrome to reveal the collagen fibres. This staining can be used to determine the degree of fibrosis or scarring brought on by long-term alcohol use in an area of liver tissue that has been exposed to ethanol.

4.15.2.1 MATERIALS

- ✚ Zebrafish liver section unstained slides,
- ✚ Xylene,
- ✚ Absolute ethanol,
- ✚ 50% ethanol,
- ✚ 25% ethanol,
- ✚ Distilled water,
- ✚ Bouin's solution (Picric acid),
- ✚ Mayer's Haematoxylin,
- ✚ Mounting medium.

4.15.2.2 PROCEDURE

Initially, the sections were deparaffinized by incubating them in xylene for 3 minutes, followed by immersion in absolute ethanol for 3 minutes, and subsequently rehydrated through a series of ethanol solutions: 50% ethanol for 3 minutes, 25% ethanol for 3 minutes, and distilled water for 3 minutes. The sections were then stained by immersing them in Bouin's solution at 56-60°C for 2 hours,

followed by thorough rinsing in running tap water until no yellow colour remained. Mayer's haematoxylin was applied for 5 minutes, followed by a rinse in running tap water. The sections were then rapidly dehydrated through a series of ethanol solutions: 50% ethanol for 30 seconds, and absolute ethanol for 30 seconds. After dehydration in absolute ethanol for 30 seconds, the sections were mounted using a DPx mounting medium and cover slip was placed over it. The slides were visualized at light microscope.



Fig:4.12 : Massons Trichome Staining Process

4.16 STATISTICAL ANALYSIS

Data were analyzed by one-way ANOVA, followed by Tukey's post-hoc unpaired test for comparisons among groups using Graph Pad Prism. Few experiments the values were compared with Student T- Test unless mentioned. Comparisons with p-values ≤ 0.05 were considered statistically significant.

CHAPTER 5

RESULTS

5.1 LEVEL OF TOTAL PROTEIN ESTIMATION

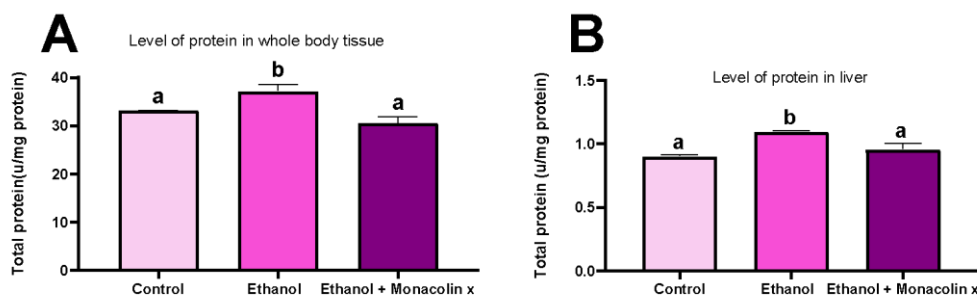


Fig:5.1 : Level of total protein in whole body tissue and liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

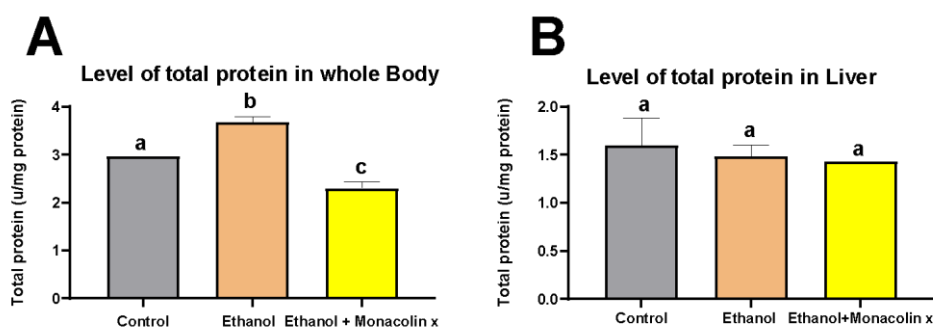


Fig:5.2 : Level of total protein in whole body tissue and liver of chronic induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

The level of total proteins were estimated from each groups (Control, 0.5% Ethanol and 0.5% Ethanol + 2 μ M Monacolin X). The Fig 5.1 represents the acute induction of zebrafish for 3 days and Fig 5.2 represents the chronic liver injury induction for 30 days. The Fig 5.1 (A) represents the level of total protein present in whole body tissue and (B) graph represents the level of total protein present in

liver of adult zebrafish. In Fig 5.1 the results of acute induction shows control group has the lowest level of protein. The ethanol group has a higher level of protein than the control group. The Monacolin X treated group has the lowest level of protein. In Fig 5.2 the results of chronic induction shows control group has the lowest level of protein. Compare to ethanol group, treated group has lowest level of protein. Increased protein synthesis resembles that chronic ethanol consumption may increase protein synthesis in the liver in an attempt to repair damaged cells whereas decreased protein synthesis resembles that chronic ethanol consumption may also decrease protein synthesis throughout the body, possibly due to malnutrition or impaired liver function. Though the treated group of Monacolin X has lower levels of protein in liver compared to ethanol alone administrated group.

5.2 LIVER MARKER ENZYMES ANALYSIS

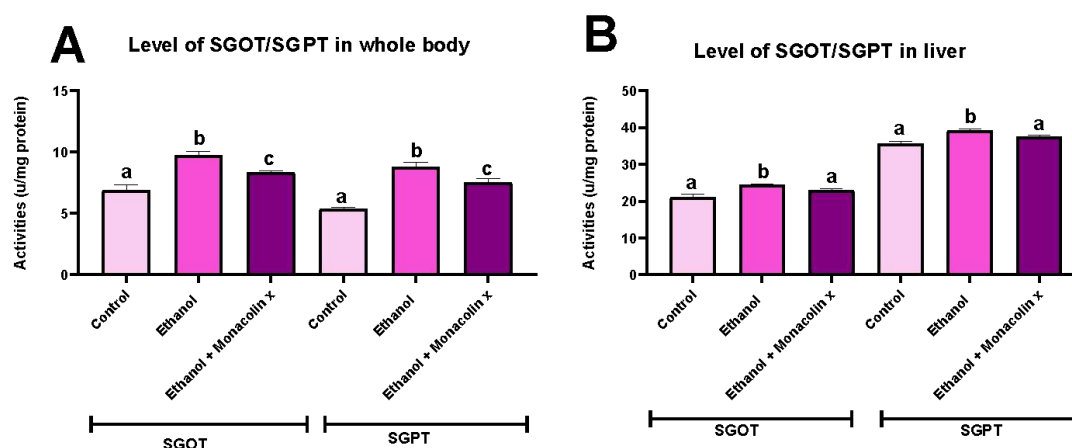


Fig:5.3 : Activities of SGOT/SGPT in blood and liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

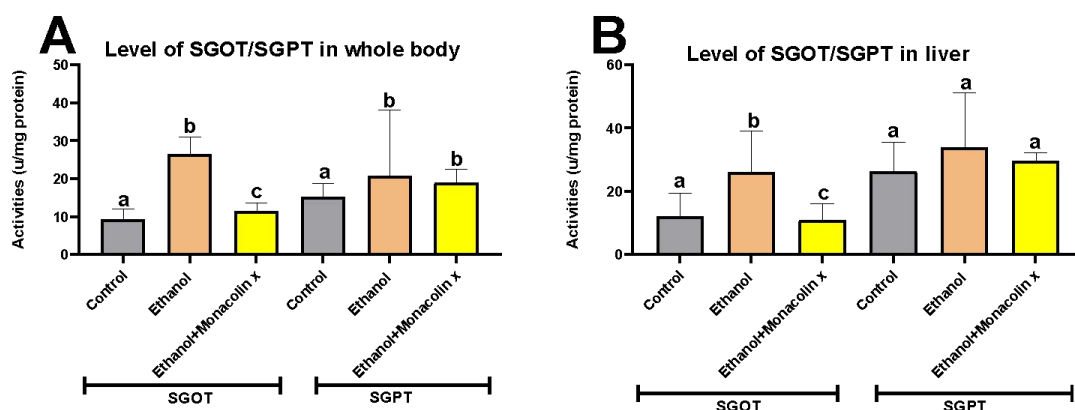


Fig:5.4 : Activities of SGOT/SGPT in blood and liver of chronic induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 8$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamic Pyruvate Transaminase (SGPT) enzymes are present in various tissues throughout the body, with particularly high concentrations in the liver. The liver cells are damaged or destroyed, SGOT and SGPT leak into the bloodstream, making them useful indicators of liver damage or disease. The SGOT assay typically measures the conversion of aspartate and alpha-ketoglutarate to oxaloacetate and glutamate. SGPT, measures the conversion of alanine and alpha-ketoglutarate to pyruvate and glutamate. These reactions are catalyzed by the respective enzymes. In Fig 5.3 the acute induction of ethanol and treated represents the activity of SGOT/SGPT enzyme in blood and liver. The treated group shows decreased activity compared to ethanol group. In Fig 5.4 the chronic induction of ethanol and treated represents the activity of SGOT/SGPT enzyme in blood and liver. The treated group results decreased activity compared to ethanol group.

5.3 BIOCHEMICAL ANALYSIS (OXIDATIVE STRESS MARKERS)

5.3.1 SUPEROXIDE DISMUTASE (SOD)

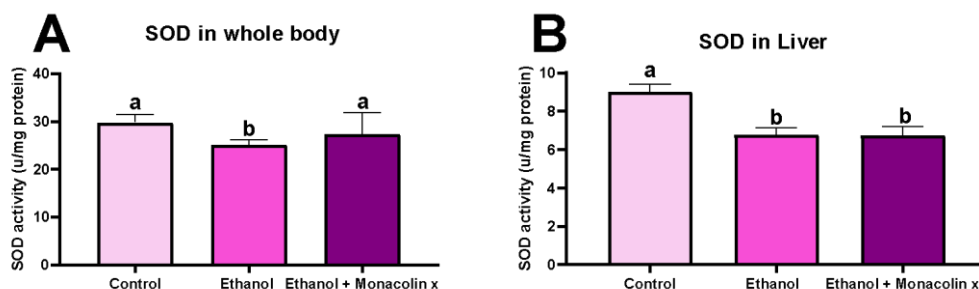


Fig:5.5 : Activity of an enzyme Superoxide Dismutase (SOD) in whole body tissue and liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

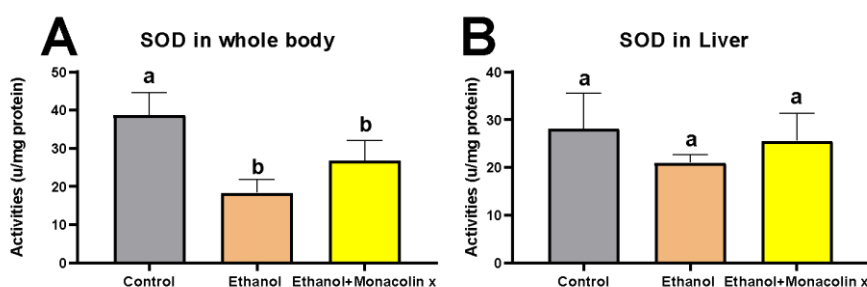


Fig:5.6 : Activity of an enzyme Superoxide (SOD) in whole body tissue and liver of chronic induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

Superoxide Dismutase (SOD) enzyme, which plays a crucial role in the defence against oxidative stress by converting superoxide radicals into hydrogen peroxide and oxygen. In Fig 5.5 the acute induction of ethanol and treated represents the activity of SOD enzyme in blood and liver. The activity of SOD enzyme is increased in treated group compared to ethanol group. In Fig 5.6 the chronic induction of ethanol and treated represents the activity of SOD enzyme in blood and liver. The treated group results increased activity compared to ethanol group.

5.3.2 CATALASE (CAT)

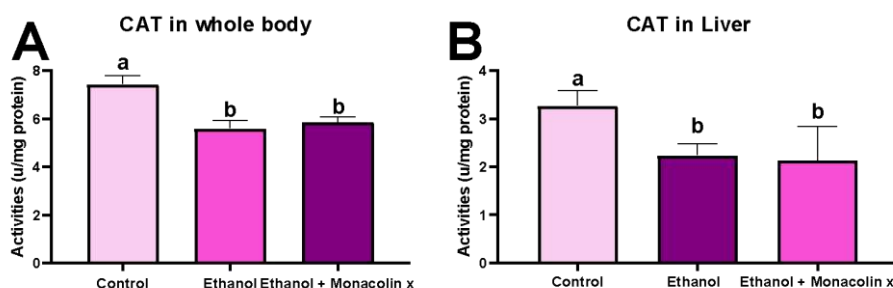


Fig:5.7 : Activity of an enzyme CAT in whole body tissue and liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM (n = 10); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

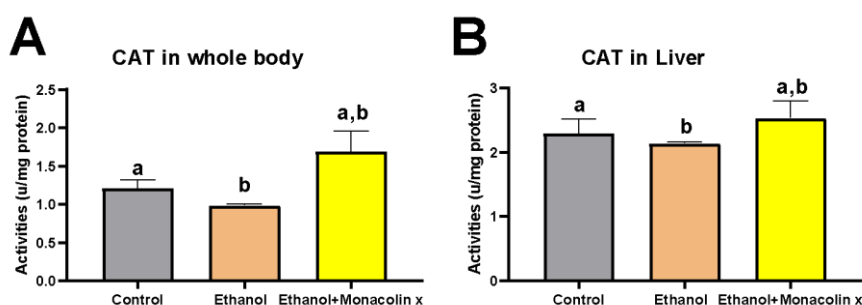


Fig:5.8 : Activity of an enzyme CAT in whole body tissue and liver of chronic induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM (n = 10); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

Catalase (CAT) is an important enzyme found that helps convert hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). This reaction is crucial for the detoxification of hydrogen peroxide, a harmful by-product of various metabolic processes, into harmless substances. In Fig 5.7 the acute induction of ethanol and treated represents the activity of CAT enzyme in whole body tissue and liver. The activity of CAT enzyme is increased in treated group compared to ethanol group. In Fig 5.8 the chronic induction of ethanol and treated represents the activity of CAT enzyme in whole body tissue and liver. The treated group results increased activity compared to ethanol group.

5.3.3 GLUTATHIONE PEROXIDE (GPx)

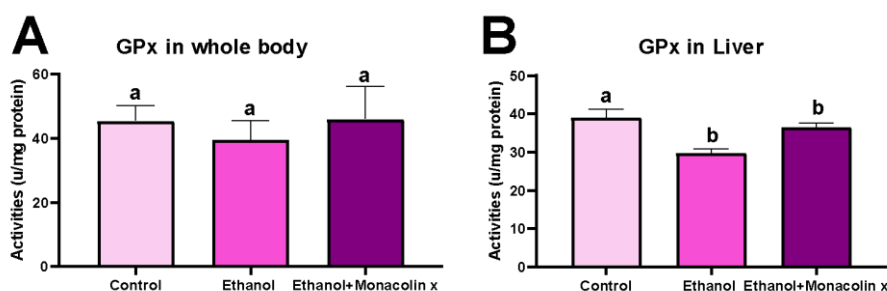


Fig:5.9 : Activity of an enzyme GLUTATHIONE PEROXIDE (GPx) in whole body tissue and liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

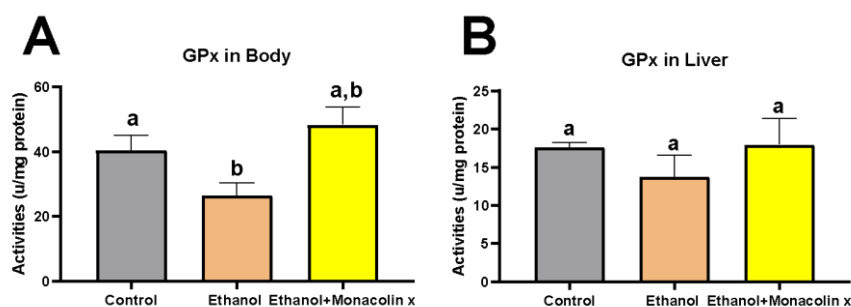


Fig:5.10 : Activity of an enzyme GLUTATHIONE PEROXIDE (GPx) in whole body tissue and liver of chronic induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

GPx is an enzyme that assesses the activity of the enzyme glutathione peroxidase, which reduces hydrogen peroxide using glutathione as a substrate. In Fig 5.9 the acute induction of ethanol and treated represents the activity of GPx enzyme in whole body tissue and liver. The activity of GPx enzyme is increased in treated group compared to ethanol group. In Fig 5.10 the chronic induction of ethanol and treated represents the activity of GPx enzyme in whole body tissue and liver. The treated group results increased activity compared to ethanol alone treated group.

5.3.4 GLUTATHIONE (GSH)

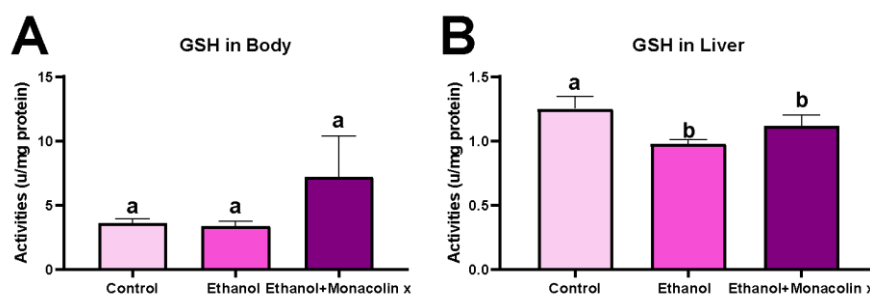


Fig:5.11 : Activity of an enzyme GLUTATHIONE (GSH) in whole body tissue and liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

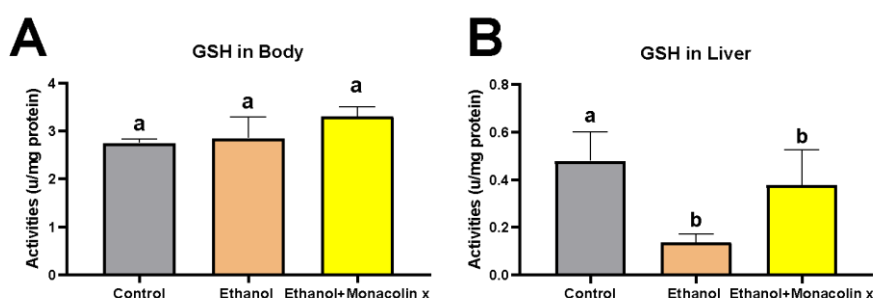


Fig:5.12 : Activity of an enzyme GLUTATHIONE (GSH) in whole body tissue and liver of chronic induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

GSH (Reduced Glutathione) estimated to measure the levels of reduced glutathione, an important antioxidant molecule. Glutathione (GSH) is oxidised to create the yellow derivative 5'-thio-2-nitrobenzoic acid (TNB) by the sulfhydryl reagent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). In the presence of NADPH, glutathione reductase can recycle the generated glutathione disulfide (GSSG) into GSH (Irfan Rahman *et al.*, 2006). In Fig 5.11 the acute induction of ethanol and treated represents the activity of GSH enzyme in whole body tissue and liver. The activity of GSH enzyme is increased in treated group compared to ethanol group. In Fig 5.12 the chronic induction of ethanol and treated represents the activity of

GSH enzyme in whole body tissue and liver. The treated group results increased activity compared to ethanol group.

5.3.5 GLUTATHIONE S TRANSFERASE (GST)

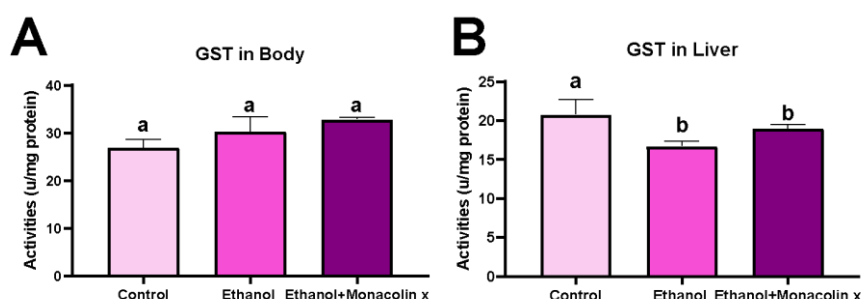


Fig:5.13 : Activity of an enzyme *GLUTATHIONE S TRANSFERASE (GST)* in whole body tissue and liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

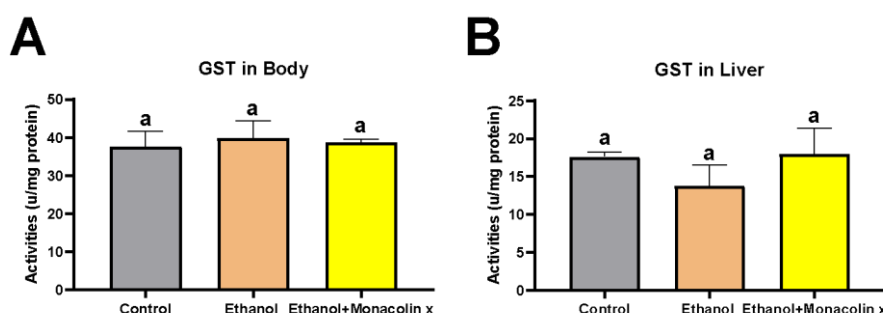
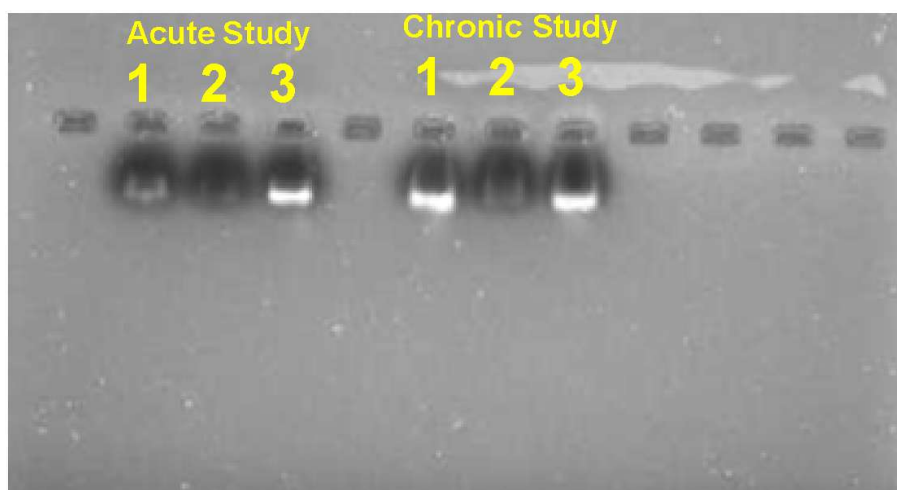


Fig:5.14 : Activity of an enzyme *GLUTATHIONE S TRANSFERASE (GST)* in whole body tissue and liver of chronic induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

GST (Glutathione-S-Transferase) used to measures the activity of the enzyme glutathione-S-transferase, which plays a role in detoxification. The activity of GSTs was measured by spectrophotometry using reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) as substrates (Lila moatamedi pour *et al.*, 2014). In Fig 5.13 the acute induction of ethanol and treated represents the activity of GST enzyme in whole body tissue and liver. The activity of GST enzyme is

increased in treated group compared to ethanol group. In Fig 5.14 the chronic induction of ethanol and treated represents the activity of GST enzyme in whole body tissue and liver. The treated group results increased activity compared to ethanol group.

5.4 AGAROSE GEL ELECTROPHORESIS



Lane 1: C = Control

Lane 2: E = 0.5% Ethanol

Lane 3: T = 0.5% Ethanol + Monacolin X

Fig:5.15 : DNA FRAGMENTATION ANALYSIS

The DNA was separated based on their size & charge in the electric field. In figure 5.15 the DNA were intact in control whereas in ethanol the DNA are fragmented due to inflammation the DNA were broke down. In treated the DNA were protected and intact DNA has shown.

5.5 QUANTITATIVE REAL-TIME-POLYMERASE CHAIN REACTION (qRT-PCR)

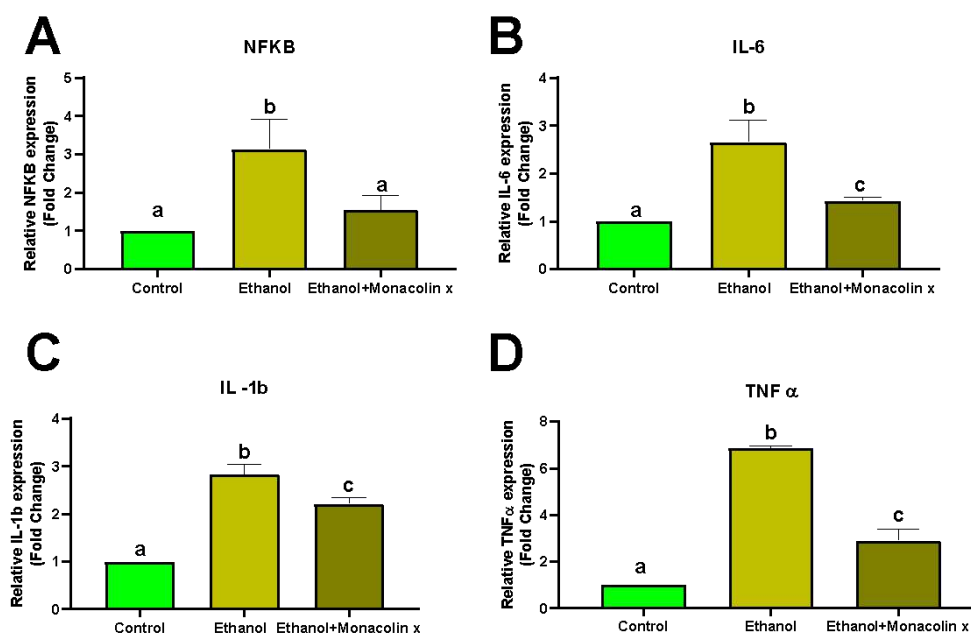


Fig:5.16 : Quantitative RT-PCR Analysis of Inflammation-Related Genes in the zebrafish of control and ethanol administrated fish for 3 days as acute liver injury study. The zebrafish treated with or without 0.5% ethanol or Monacolin X. Values represent the mean $\pm$ SEM of 5-8 animals. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$. (as determined by Tukey's test based on repeated two-way ANCOVA).

The Δ CT value greater than 1 suggests that the expression of inflammatory markers such as Nuclear Factor kappa B (NFkB), Interleukins-6 (IL-6), Interleukins-1 β (IL-1 β) and Tumour Necrosis Factor alpha (TNF α) genes increased when zebrafish were treated with ethanol administration. This indicates that ethanol treatment might have induced inflammation, as NFkB induces the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines, and also participates in inflammation regulation, IL-6 is a typical immune-modulating cytokine with a potential pro-inflammatory ability that stimulates both the innate and adaptive immune systems, IL-1 β is a key mediator of the inflammatory response and TNF α is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation (Baolin Xu *et al.*, 2020). The

Δ CT value is less than that of ethanol treatment but still higher than the control. It suggests that the addition of Monacolin X reduced the expression of inflammatory markers compared to ethanol treatment alone (Nagabhishek, S.N. *et al.*, 2019). These reduction indicates that Monacolin X might have mitigated the inflammatory response induced by ethanol. Ethanol treatment alone appears to increase the expression of inflammatory markers, indicating an induction of inflammation. However, when Monacolin X was added along with ethanol, the expression of Nuclear Factor kappa B (NFkB), Interleukins-6 (IL-6), Interleukins-1 β (IL-1 β) and Tumour Necrosis Factor (TNF α) were decreased compared to ethanol treatment alone. This suggests that Monacolin X might have anti-inflammatory effects, as it reduced the expression of Nuclear Factor kappa B (NFkB), Interleukins-6 (IL-6), Interleukins-1 β (IL-1 β) and Tumour Necrosis Factor (TNF α) genes induced by ethanol alone treated groups.

5.6 HISTOPATHOLOGY RESULTS

The Haematoxylin & Eosin stained slides were observed in light microscope at 40x magnification of Control, 0.5% Ethanol and 0.5% Ethanol+Monacolin X. The fat accumulation in liver were visualized in light microscope after Haematoxylin & Eosin staining. Usually, hepatocytes (liver cells) containing small, clear vacuoles within their cytoplasm, indicates the presence of fat droplets. The unstained slides were stained using Masson's trichome staining were also visualized in light microscope at 40x magnification and the results are shown below.

5.6.1 HAEMATOXYLIN & EOSIN STAINING OF ACUTE INDUCTION

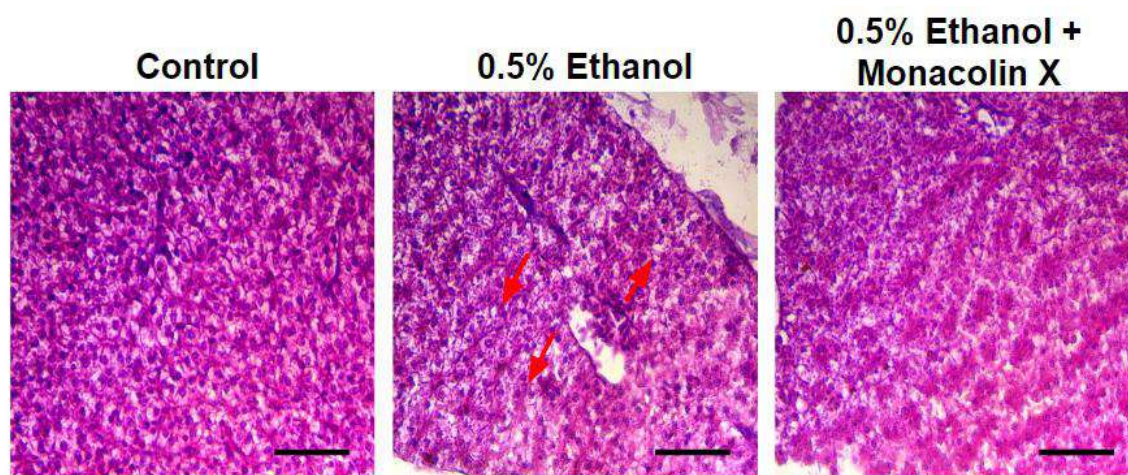


Fig:5.17 : The Haematoxylin and Eosin (H&E) staining of liver sections from zebrafish treated with Monacolin X for 3 days. Photomicrograph of representative H&E stained liver sections of control, ethanol, and Monacolin X-supplemented in zebrafish. Micro and macrovesicular steatosis was observed in liver sections of ethanol fed zebrafish, while liver sections of Monacolin X supplemented ethanol fish liver showed microvesicular steatosis. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter differ significantly from each other at $p \leq 0.05$. Scale bar = 50 μm (Magnification 40X).

In zebrafish, acute treatment with 0.5% Ethanol and 0.5% Ethanol + Monacolin X induces micro steatosis, which refers to the accumulation of small fat droplets in liver cells, which is implicated by the deposition of round droplets in the hepatocytes. Normal staining patterns observed in tissue areas without microsteatosis, with pink-stained cytoplasm and blue-purple nuclei. Fig 5.16 The hepatocytes containing microsteatosis are designated with red marking arrows. (Sampey, B.P. *et al.*, 2003). Therefore treated Monacolin X reveals normal morphology compared to ethanol induction.

5.6.2 HAEMATOXYLIN & EOSIN STAINING OF CHRONIC INDUCTION RESULT

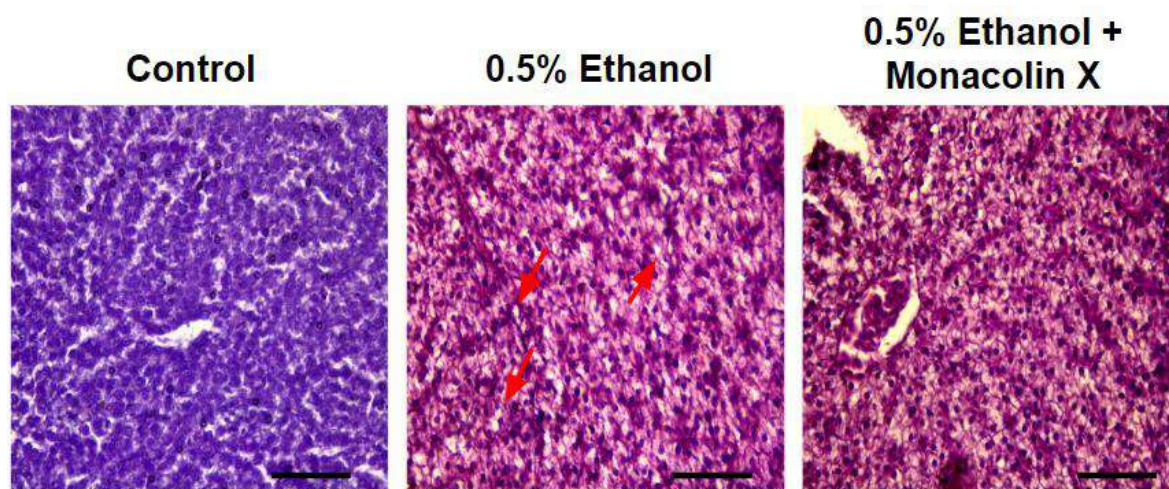


Fig:5.18 : The Haematoxylin and Eosin (H&E) staining of liver sections from zebrafish treated with Monacolin X. The Haematoxylin and Eosin (H&E) staining of liver sections from zebrafish treated with Monacolin X for 30 days. Photomicrograph of representative H&E stained liver sections of control, ethanol, and Monacolin X supplemented in zebrafish. Micro and macrovesicular steatosis was observed in liver sections of ethanol fed zebrafish, while liver sections of Monacolin X supplemented ethanol fish liver showed microvesicular steatosis. Data are presented as the mean $\pm$ SEM ($n = 10$); values not sharing a common letter differ significantly from each other at $p \leq 0.05$. Scale bar = 50 μm (Magnification 40X).

In zebrafish, chronic treatment with 0.5% Ethanol and 0.5% Ethanol+Monacolin X induces steatosis, which refers to the accumulation of small fat droplets in liver cells, which is implicated by the deposition of round droplets in the hepatocytes. Normal staining patterns observed in tissue areas without microsteatosis, with pink-stained cytoplasm and blue-purple nuclei. Normal architecture with clear visible of Hepatic portal vein & organised cellular Hepatocytes were observed in control. In Fig 5.16 the hepatocytes containing steatosis are designated with red marking arrows in ethanol induction. The ethanol treated reveals steato hepatitis which is higher in fat accumulation. Therefore, treated Monacolin X visualized a normal morphology compared to ethanol induced.

5.6.3 MASSONS TRICHROME STAINING OF ACUTE INDUCTION RESULT

Tissue samples were stained with Masson's Trichrome to reveal the collagen fibres. This staining can be used to determine the degree of fibrosis or scarring brought on by long-term alcohol use in an area of liver tissue that has been exposed to ethanol. Masson's Trichrome procedure, usually explores the collagen deposition in the hepatocytes are stained blue, nuclei are stained dark brown, liver tissue is stained red, and cytoplasm is stained pink.

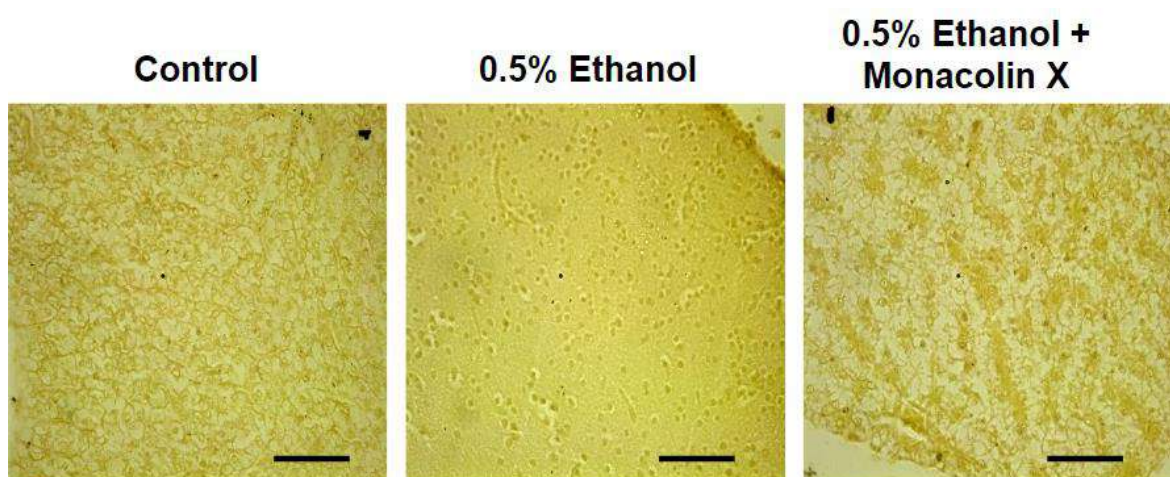


Fig:5.19 : The Masson's trichrome staining for collagen in of liver sections from zebrafish treated with Monacolin X. The fishes are administrated with 0.5% Ethanol for 3 days as acute liver injury study. Normal liver (Control), 0.5% ethanol administration showed pericentral fibrosis and early deposition of fine collagen fibers in middle image. Well developed hepatic fibrosis and early cirrhosis. Deposition of extensive thick collagen fibers in the hepatic parenchyma. Monacolin X treatment with ethanol administration shows the formation of bridging fibrosis between central vein and portal triads and deposition of thick collagen fibers. Original magnification, 40X (Scale bar =50µm).

The higher inflammation was shown in ethanol induction compare to ethanol Monacolin X has lowered the fat accumulation.

5.6.4 MASSON'S TRICHROME STAINING OF CHRONIC INDUCTION RESULT

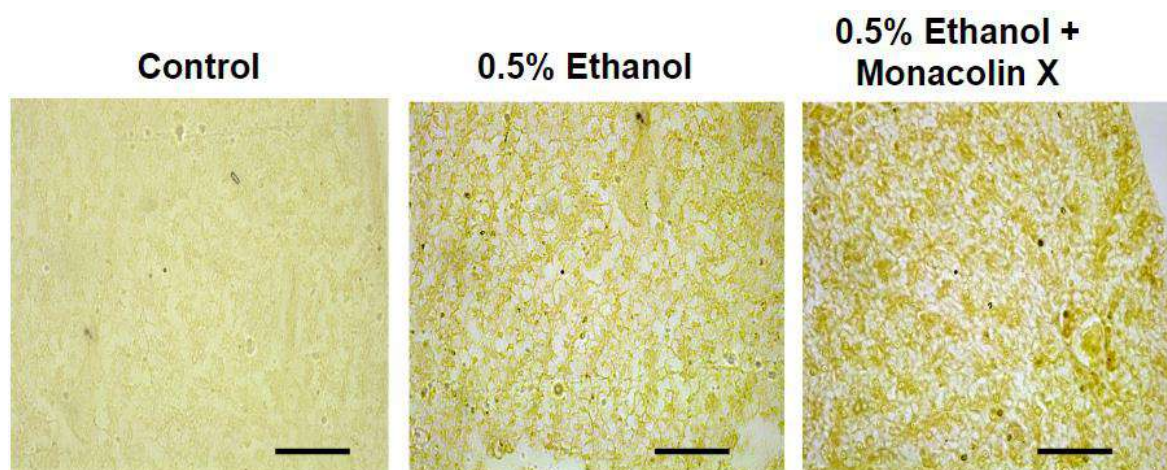


Fig:5.20 : The Masson's trichrome staining of liver sections from zebrafish treated with Monacolin X. The Masson's trichrome staining for collagen in of liver sections from zebrafish treated with Monacolin X. The fishes are administrated with 0.5% Ethanol for 30 days as chronic liver injury study. Normal liver (Control), 0.5% ethanol administration showed pericentral fibrosis and early deposition of fine collagen fibers in middle image Well developed hepatic fibrosis and early cirrhosis. Deposition of extensive thick collagen fibers in the hepatic parenchyma. Monacolin X treatment with ethanol administration shows the formation of bridging fibrosis between central vein and portal triads and deposition of thick collagen fibers. Original magnification, 40X (Scale bar =50 μ m).

The control has shown no inflammation where the chronic ethanol induction reveals steatosis, treated with Monacolin X has lower fat droplets compared to ethanol. Therefore, the Monacolin X acts as a protective compound against fat deposits are due to ethanol consumption. Hence, this results showed that monacolin X actively participate in the regulation inflammatory mediators, which indicating its anti-inflammatory activity.

DISCUSSION

The previous studies were reported that the ethanol-induced acute liver injury model in adult zebrafish (Ki-Hoon Park *et al.*, 2020) (Changwen Zhang *et al.*, 2016) (Haiyan Lin *et al.*, 2017). Crucially a model simply needed to exposure to the treatment with the zebrafish either ethanol or Monacolin X for three days in order to evaluate a drug's effectiveness. This means that it might be used for high-throughput *in vivo* drug screening in order to find novel medications that could shield the liver from acute hepatic injury caused by ethanol (Benic, M.S. *et al.*, 2022). With this model system, they discovered that acute hepatic damage due to ethanol exposure was totally inhibited by riboflavin, TES-1025, or NR supplementation. Their findings also raised the intriguing possibility that hepatocytes protected from ethanol-induced acute hepatic injury could prevent the development of alcoholic steatohepatitis, and that dietary supplementation of specific compounds during binge or heavy alcohol drinking might lessen the severity of hepatic injury (Park, K.-H. & Kim, S.-H. 2020).

Alcohol-induced acute and chronic hepatic injury models are particularly valuable for rapid screening of drugs that may offer therapeutic benefits for ALD associated with acute hepatic injury caused by excessive alcohol consumption. In this study, 0.5% ethanol showed elevated SGOT and SGPT levels in blood and liver tissue of the experimental zebrafishes. Thus, indicating liver damage from an ethanol administration. Similarly, Ki-Hoon Park have reported that 0.5% ethanol administration reflected the increased levels of SGOT and SGPT in liver and serum of control and experimental zebrafish (Park, K.-H. & Kim, S.-H. 2020). Additionally, Fish fed *F. benghalensis* supplemented feed showed a small increase in these enzymes although value dropped within typical physiological bounds. Similar results for these enzymes were also noted in *Labeorohita* larvae. Michael J Passeri *et al.*, reported that zebrafish exposing 4 dpf zebrafish larvae to 2% ethanol for 32 hours achieves approximately 80mM intracellular Ethanol and up-regulation of hepatic cyp2e1, sod, and bip, indicating that Ethanol is metabolized and provokes oxidant stress (Passeri, M.J. *et al.*, 2008). The study concludes by showing that following exposure for seven to fourteen days, BP-4, PABA, and PBSA may significantly alter SOD, GST, GSH, and MDA in zebrafish liver. These observations suggest that fish liver oxidative injury and increased ROS generation

may result from exposure to these organic UV filters (Huang, X. *et al.*, 2020). SOD, as the first line of defense against oxidative stress, can catalyze the mutation of O_2^- and convert it into H_2O and H_2O_2 . GST, a phase II detoxification metabolic enzyme, can catalyze the binding of electrophilic groups of xenobiotics with sulfhydryl groups of GSH to increase its hydrophobicity (Huang, X. *et al.*, 2020). GST also has GPx activity, which can inhibit lipid peroxidation. In this experiment, compared with the control group, SOD activity decreased significantly in all the groups after 7 days of exposure to three chemicals. Excessive ROS production in fish after exposure, which exceeds the ability of SOD to remove ROS, was a cause of the results (Tao, Y. *et al.*, 2013). Similarly, in our study the treatment with Monacolin X shown alter antioxidants such as SOD, GST and GSH in zebrafish liver.

Additionally, Sears et al. hypothesised that ethanol's effects on inflammatory mediators varied depending on the tissue. After a bilateral femur fracture with fixation in model, they discovered that acute alcohol consumption reduced the levels of inflammation biomarkers (such as IL-2, IL-6, and IL-10) in blood while increasing the levels of IL-2, IL-6, IL-1 β , and macrophage inflammatory protein-1 α in the protein. Chronic alcohol administration reversed the effects on proinflammatory cytokines in patients with a history of alcoholism, leading to increased levels of TNF α , IL-1 β , and IL-6. In an *in vitro* experiment comparing acute and chronic alcohol use, acute alcohol led to an increase in IL-10 production in human monocytes and a decrease in TLR8 or TLR4-induced TNF- α protein and mRNA expressions. Nonetheless, persistent alcohol consumption increased TNF- α 's protein level and mRNA without changing the IL-10 production in monocytes. (Baolin Xu *et al.*, 2020). The findings present in our study highlights the complex interplay between ethanol exposure and inflammation, as well as the potential therapeutic benefits of Monacolin X in mitigating inflammatory responses. Ethanol is known to induce inflammation through various mechanisms, including the activation of NF κ B and the upregulation of pro-inflammatory cytokines such as IL-6, IL-1 β , and TNF α . These inflammatory responses can contribute to tissue damage and disease progression.

In conclusion, the findings from this project shed light on the promising protective effects of Monacolin X against acute and chronic ethanol-induced hepatic

steatosis and injury. Through meticulous experimentation and analysis, we have demonstrated the potential of Monacolin X as a therapeutic agent in mitigating the detrimental effects of ethanol on the liver. These results not only contribute to our understanding of the pathophysiology of hepatic steatosis but also offer a beacon of hope for the development of novel treatments for alcohol-induced liver diseases. Moving forward, further research and clinical trials are warranted to fully elucidate the mechanisms underlying Monacolin X's efficacy and to validate its therapeutic potential in pre-clinical and clinical settings. This project serves as a crucial stepping stone in the ongoing pursuit of effective interventions to safeguard liver health and combat the devastating consequences of alcohol abuse.

CHAPTER 6

SUMMARY & CONCLUSION

This present study examined Monacolin X as a potential effect against chronic ethanol-induced liver damage in zebrafish. The results convincingly demonstrate that Monacolin X administration offers significant advantages in countering these harmful effects. Monacolin X balanced protein levels, potentially preventing the liver's repair mechanisms from becoming overloaded due to chronic ethanol exposure. Lower liver enzyme activities (SGOT/SGPT) indicated improved liver function and less injury in the Monacolin X treated group compared to the ethanol group. Monacolin X significantly boosted the activity of antioxidant enzymes such as SOD, CAT, GPx, GST, GSH. This effectively combats oxidative stress and safeguards cells from damage in the ethanol-exposed zebrafish.

Monacolin X treatment protected DNA from fragmentation, a consequence of inflammation triggered by chronic ethanol consumption. Monacolin X exhibited anti-inflammatory properties by downregulating the expression of inflammatory genes such as NF- κ B, IL-6, IL-1 β , TNF- α . This suggests its potential to lessen the inflammatory response induced by ethanol. Finally, histopathological analysis revealed that Monacolin X treatment prevented fat accumulation in the liver (steatosis) observed in the ethanol group. Masson's trichrome staining further supported this, demonstrating reduced inflammation and lower fat deposits in the Monacolin X treated group.

In conclusion, this study provides compelling evidence that Monacolin X administration offers a promising therapeutic approach to protect against chronic ethanol-induced hepatic steatosis and injury. It reduces protein synthesis, improves liver function, enhances antioxidant defense, preserves DNA integrity, mitigates inflammation, and prevents fatty liver disease. Further research is necessary to confirm these findings in clinical and pre-clinical and assess the safety and efficacy of Monacolin X for human use in managing alcoholic liver disease.

ANNEXURE

	SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY (DEEMED TO BE UNIVERSITY) Category -1 University by UGC Accredited with "A++" Grade by NAAC Chennai – 600 119, Tamil Nadu, India		
INSTITUTIONAL ANIMAL ETHICS COMMITTEE			
CERTIFICATE			
This is to certify that the project proposal no. SU/CLATR/IAEC/XXII/29/2024 entitled "Protective effect of monacolin against chronic ethanol induced hepatic steatosis" submitted by Dr. A. Madan kumar has been approved by the IAEC of Sathyabama Institute of Science and Technology, Chennai 600119 in its meeting held on 02nd March 2024 and 40 numbers of <i>Danio rerio</i> (Zebra Fish) have been sanctioned under this.			
Authorized by	Name	Signature	Date
Chairman	Dr. B. Sheela Rani		13/03/24
Member Secretary	Dr. D. Saravanan		13/03/24
Main Nominee of CCSEA	Dr. D. Ezhilarasan		13/03/24

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