

**HEPATOPROTECTIVE ROLE OF CYCLO(-PRO-TYR) AGAINST
ALCOHOL-ASSOCIATED LIVER DISEASE IN ZEBRAFISH
(*Danio rerio*)**

PROJECT WORK

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

By

HENCY MATILDA T J (41770010)



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

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

CATEGORY -1 UNIVERSITY BY UGC

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JEPPIAR NAGAR, RAJIV GANDHI SALAI CHENNAI-600119

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **HENCY MATILDA T J (41770010)** who carried out the project entitled "**HEPATOPROTECTIVE ROLE OF CYCLO(-PRO-TYR) AGAINST ALCOHOL-ASSOCIATED LIVER DISEASE IN ZEBRAFISH (*Danio rerio*)**" under my supervision from January 2024 to April 2024.

Internal Guide

Dr. S. JAYASHREE

External Guide

Dr. A. MADAN KUMAR

Head of the Department

Dr. V. RAMESH KUMAR

Submitted for Viva-voce Examination held on 26/4/24

Internal Examiner

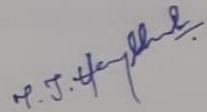
External Examiner

DECLARATION

I HENCY MATILDA T J (41770010) hereby declare that the Project Report entitled "HEPATOPROTECTIVE ROLE OF CYCLO(-PRO-TYR) AGAINST ALCOHOL-ASSOCIATED LIVER DISEASE IN ZEBRAFISH (*Danio rerio*) "done by me under the guidance of Dr. A. MADAN KUMAR, Associate professor (Research), International Research Centre, Sathyabama Institute of Science and Technology is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in Biotechnology.

DATE: 26-04-2024

PLACE: CHENNAI



SIGNATURE OF THE CANDIDATE

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ABSTRACT

The liver is known for its remarkable regenerative abilities, it is a vital hub for various physiological functions. Liver disease contributes to over 2 million deaths annually. Chronic alcohol abuse is common and a leading cause of alcoholic liver disease. The utilization of zebrafish as an animal model has increased greatly in recent years due to its unique advantages such as rapid disease progression, cost-effectiveness and many more reasons. In this study, the utility of adult zebrafish as an in vivo model for rapidly assessing cyclo(-pro-tyr) efficacy in ethanol-induced acute liver disease was evaluated. The adult zebrafish were exposed to 0.5% ethanol for 3 days. There was an elevation in SGOT and SGPT in the ethanol treatment group compared to the control group. The cyclo(-pro-tyr) treatment group resulted in the suppression of SGOT and SGPT. Biochemical analyses of antioxidants in the liver and whole tissue showed a decrease in antioxidant activity in the ethanol-induced group compared to the control. In the cyclo(-pro-tyr) groups the activity was seen to be increased. Additionally, there was an upregulation in the mRNA expression of inflammatory marker genes.

Keywords: Acute liver disease, Cyclo(-pro-tyr), Ethanol-induced, Inflammation, Liver, Zebrafish

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LIST OF ABBREVIATIONS

ADHs	Alcohol dehydrogenases
ALD	Alcohol-associated liver disease
ALF	Acute liver failure
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUD	Alcohol use disorder
BSA	Bovine serum albumin
CAT	Catalase
cDNA	Complementary DNA
CDNB	1-Chloro-2,4-dinitrobenzene
CLD	Chronic liver disease
DILI	Drug-induced liver injury
DNA	Deoxyribonucleic acid
DNPH	2,4-Dinitrophenylhydrazine
dpf	days post-fertilization
DTNB	5'-dithiobis (2-nitrobenzoic acid)
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
EtBr	Ethidium Bromide
EtOH	Ethanol
GPx	Glutathione peroxide
GSH	Glutathione
GST	Glutathione S-transferase
H&E	Hematoxylin & Eosin
HBV	Hepatitis B
HCC	Hepatocellular carcinoma
HCV	Hepatitis C
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
MAFLD	Metabolic-associated liver disease
mbtps1	membrane-bound transcription factor protease 1

MgIG	Magnesium Isoglycyrrhizinate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-kB	Nuclear factor kappa B
PBS	Phosphate buffer saline
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
scap	SREBP cleavage-activating protein
SDS	Sodium dodecyl sulphate
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SOD	Superoxide dismutase
SPEE	Salvia plebeia R. Br
SREBPs	Sterol regulatory element binding proteins
TBE buffer	Tris borate EDTA
TCA	Trichloroacetic acid
TNF- α	Tumour Necrosis Factor- α

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CHAPTER 1

INTRODUCTION

1.1 LIVER

The liver is a vital organ with remarkable natural regenerative abilities, capable of restoring lost tissue, with approximately 25% of its mass able to regenerate into a fully functional organ (Neshat *et al.*, 2021; Sheedfar *et al.*, 2013). It is among the most crucial organs responsible for overseeing homeostasis in the mammalian body (Di-Iacovo *et al.*, 2023). The liver serves as a vital center for a multitude of physiological functions. These encompass the metabolism of macronutrients, regulation of blood volume, support for the immune system, control of growth signaling pathways through endocrine mechanisms, maintenance of lipid and cholesterol balance, and the breakdown of xenobiotic compounds, which includes the metabolism of many drugs. Among these functions, the processing, partitioning, and metabolism of macronutrients stand out as particularly crucial, as they supply the necessary energy to drive the aforementioned physiological processes. Additionally, the liver's capability to store glucose as glycogen during feeding and produce glucose via the gluconeogenic pathway in reaction to fasting is crucial. The liver metabolizes lipids and packages any excess lipids for transportation and storage in other tissues like adipose tissue. Additionally, it plays a central role in regulating protein and amino acid metabolism, being responsible for the secretion of the majority of proteins into the bloodstream, converting amino acids into energy, and eliminating nitrogenous waste from protein breakdown through urea metabolism. Throughout the evolutionary process, these diverse functions have become consolidated within the liver, a vital organ found in all vertebrates. Developmentally, the liver undergoes a complex differentiation program initiated by external signals, cellular cues, and a network of transcription factors. These processes, essential for life, are fully established in the mature liver (Trefts *et al.*, 2017). The liver is comprised of diverse cell types, originating from distinct embryological origins, including hepatocytes, biliary epithelial cells (cholangiocytes), stellate cells, Kupffer cells, and liver sinusoidal endothelial cells. Each of these cell types possesses unique functions that collaboratively regulate hepatic activity across various levels. Hepatocytes, representing the primary epithelial cell

population within the liver, constitute most of its volume and undertake many essential liver functions (Trefts *et al.*, 2017).

1.2 Liver diseases

1.2.1 Types of liver disease based on cause

1.2.1.1 Alcohol-associated liver disease

Liver disease generally refers to conditions that damage the liver. Alcohol-associated liver disease (ALD) is liver damage due to excessive alcohol consumption. It involves a broad spectrum of diseases such as steatosis, steatohepatitis, hepatitis, cirrhosis, and hepatocellular carcinoma (Liu *et al.*, 2021).

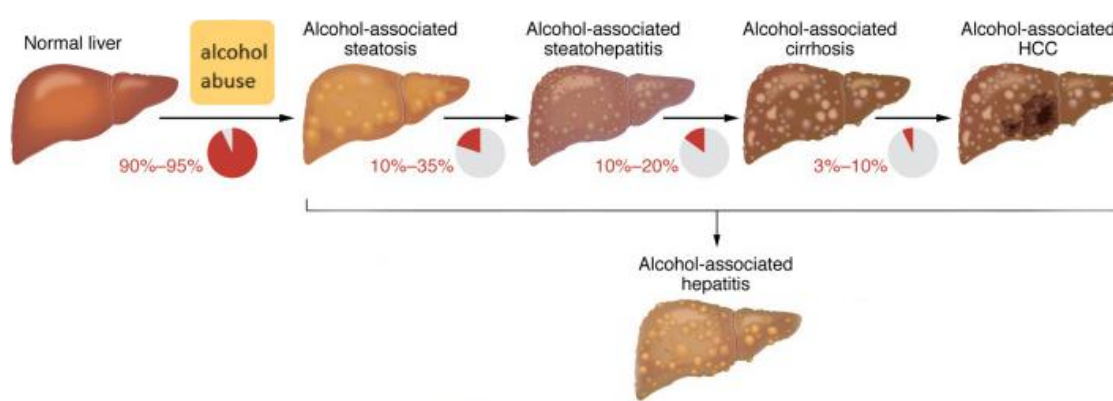


Fig.1.1: Spectrum of alcohol-associated liver disease (Mackowiak *et al.*, 2024)

Its progression is associated with the amount and duration of alcohol consumption. Upon ingestion of ethanol, alcohol dehydrogenases (ADHs) enzymes oxidize it to acetaldehyde. The liver plays a pivotal role in this metabolic process, converting acetaldehyde to acetate. However, this pathway operates relatively slowly compared to other biological processes (Tilg *et al.*, 2011). Acetaldehyde, a carcinogenic compound, poses a significant threat to liver health and is a primary contributor to ALD. ALD can lead to the formation of adducts, chemical modifications that may disrupt normal biological functions. Moreover, the oxidation of

acetaldehyde to acetate generates reactive oxygen species (ROS) as byproducts and excessive accumulation of these molecules induces oxidative stress, which is believed to play a critical role in ALD (Neshat *et al.*, 2021). In terms of immune response, ALD is characterized by inflammation (Szabo *et al.*, 2010).

1.2.1.2 Non-alcoholic fatty liver disease

Another type is a non-alcoholic fatty liver disease (NAFLD) or Metabolic associated liver disease (MAFLD) the most prevalent liver condition, affecting approximately one-third of the global population. An individual's daily dietary intake and level of physical activity play a crucial role in determining their susceptibility to developing MAFLD, as these factors often correlate with body weight. The onset of this disease typically occurs through a two-step process. Firstly, hepatic steatosis develops, characterized by the accumulation of triglycerides within hepatocytes. Subsequently, oxidative stress and activation of proinflammatory cytokines occur, ultimately leading to fibrosis (Tiniakos *et al.*, 2010). This also has a similar spectrum of disease to ALD, steatosis, steatohepatitis, hepatitis, cirrhosis, and hepatocellular carcinoma (Singh *et al.*, 2017). It is characterized by the buildup of lipids within the liver. When oxidative stress and inflammation are present, MAFLD can progress to nonalcoholic steatohepatitis (NASH).

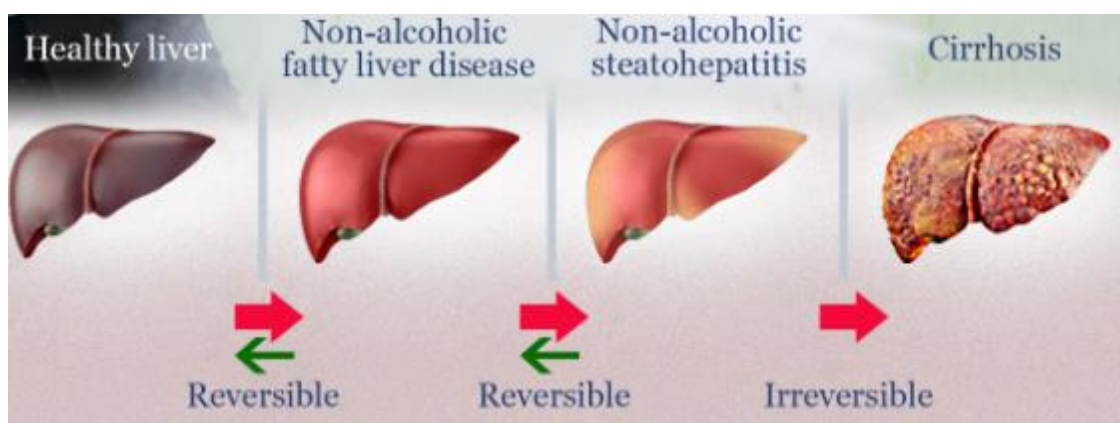


Fig:1.2: Non-alcoholic fatty liver disease (Mesnage *et al.*, 2016)

Left untreated, NASH may increase the risk of severe liver disorders such as cirrhosis, an irreversible condition marked by scarring of liver tissue, which can

potentially culminate in hepatocellular carcinoma (HCC), both of which can be debilitating or fatal. Additionally, advancing age appears to heighten the likelihood of developing MAFLD, NASH, and ultimately HCC. Although the disease is reversible during its initial phases, treatment becomes increasingly challenging as it progresses to advanced stages (Sheedfar *et al.*, 2013).

1.2.2 Types of liver disease based on persistence

Severe compromise of the liver leads to the disruption of homeostasis, which can result in various injuries. Various forms of injuries influence the occurrence of acute and chronic liver diseases.

1.2.2.1 Acute liver disease

In cases of rapid and unexpected hepatic damage, acute liver injury may occur if the liver's response is insufficient. This acute damage is characterized by an increase in infiltrating and activated neutrophils, oxidative stress, and extensive liver cell death (Jaeschke *et al.*, 2006; Lefkowitz *et al.*, 2016). Excessive loss of hepatocytes can lead to tissue degeneration, resulting in acute liver failure (ALF) (McDowell *et al.*, 2010). Various factors can cause ALF, with drug-induced liver injury (DILI) being the most frequent cause. The primary reason for DILI is an adverse reaction during the liver's metabolic process of xenobiotic biotransformation (Chen *et al.*, 2015). Furthermore, acute liver failure can arise from various causes, including viral hepatitis, which can lead to prolonged damage, and hepatic ischemia, which may occur during reperfusion following liver resection surgery. Fortunately, in the majority of cases, the reparative process triggered after the onset of liver injuries tends to resolve (Di-Iacovo *et al.*, 2023). In developed nations, paracetamol overdose stands as the primary cause of ALF, accounting for around 55% of cases. Conversely, this cause is notably rare in Asia and Africa (Rumack *et al.*, 2002).

1.2.2.2 Chronic liver disease

The liver damage persists for more than six months, it is classified as chronic liver disease (CLD). Clinically, CLD involves a cycle of liver injury and regeneration,

resulting in gradual fibrosis that may progress to cirrhosis (Schuppan *et al.*, 2008). The onset and advancement of chronic liver disease involve various factors, including viral infections, environmental influences, and genetic predispositions. Major contributors to CLD include hepatitis B (HBV) and C (HCV) viruses, excessive alcohol consumption, and obesity (Powell *et al.*, 2000).

1.2.3 Risk Factors for ALD

The development of acute and chronic liver disease due to alcohol consumption is influenced by various factors. Firstly, there needs to be a certain 'threshold' regarding the duration of alcohol use and daily intake (Thun *et al.*, 1997). However, even upon reaching this threshold, only a fraction of heavy, long-term alcohol consumers—approximately 10–35%—will develop alcoholic hepatitis, and 8–20% will develop cirrhosis (Espinoza *et al.*, 1987). Host characteristics such as gender and variations in alcohol-metabolizing enzymes, as well as external factors like obesity and hepatitis C infection, collectively determine the likelihood of developing associated liver diseases. For instance, the risk of developing HCC increases fivefold with a daily alcohol consumption of 80 g. In the presence of hepatitis C infection, this risk is increased 20-fold. Moreover, the combination of both risk factors results in a more than 100-fold increased risk for the development of HCC (Mueller *et al.*, 2009).

1.3 LIVER DISEASE INCIDENCE

Liver disease contributes to over two million deaths annually, encompassing fatalities from cirrhosis, viral hepatitis, and liver cancer. This accounts for approximately 4% of all deaths worldwide, equating to 1 out of every 25 deaths (Asrani *et al.*, 2019). Notably, one-third of liver-related deaths occur among females. Within this statistic, liver cancer alone is responsible for 600,000 to 900,000 deaths annually (Asrani *et al.*, 2019). It's important to note that liver disease may be underestimated as a cause of death. Currently, liver disease ranks the eleventh-leading cause of death globally (Griffin *et al.*, 2023). Cirrhosis specifically ranks as the tenth-leading cause of death in Africa (rising from thirteenth in 2015), the ninth-

leading cause in Southeast Asia and Europe, and the fifth-leading cause in the Eastern Mediterranean region (Devarbhavi *et al.*, 2023).

1.3.1 Alcohol-associated liver disease

In 2016, the global annual per capita consumption of alcohol was 6.4 liters per person aged 15 years or older (Rossow *et al.*, 2021). The global prevalence of alcohol use disorder (AUD) is estimated to be 5.1%, equivalent to 283 million people. The highest prevalence rates are observed among men and women in the European Region (14.8% and 3.5%, respectively) and the Americas (11.5% and 5.1%, respectively). Notably, AUD tends to be more common in high-income countries and is likely underreported and underdiagnosed in low-income nations (Devarbhavi *et al.*, 2023). Alcohol consumption significantly elevates the risk of mortality related to liver disease by 260-fold, cardiovascular mortality by 3.2-fold, and cancer mortality by 5.1-fold (Hagström *et al.*, 2021). Globally, alcohol stands as the primary cause of cirrhosis, contributing to nearly 60% of cirrhosis cases in Europe, North America, and Latin America. Additionally, approximately 35% of individuals diagnosed with AUD will develop various forms of ALD. Importantly, countries or regions with higher rates of heavy alcohol consumption tend to exhibit a greater percentage of cirrhosis cases (Stein *et al.*, 2016). In recent years, there has been a notable rise in the global incidence of alcohol-associated hepatitis, particularly among young individuals. This trend is concerning as it predominantly affects individuals aged 15-44, impacting them during their most productive years of life. This observation aligns with the finding that regular alcohol consumption during late adolescence significantly heightens the risk of alcohol-associated cirrhosis (Devarbhavi *et al.*, 2023). Alcohol consumption also heightens the risk of liver cancer, particularly in individuals who are overweight, obese or have cirrhosis related to NAFLD. In the United States, alcohol stands as the primary cause of waitlisting for liver transplantation among males and ranks second after NAFLD for females (Younossi *et al.*, 2021).

1.3.2 Metabolic-associated fatty liver disease (MAFLD)

MAFLD affects 25% of the adult population worldwide. The overall prevalence of MAFLD globally is estimated at 32.4% and has been increasing. MAFLD prevalence ranges from 24-48% in North America and is ethnicity-dependent. South America's rates are around 30%, possibly underestimated. Europe's prevalence is 23%-33%, higher in Western than Eastern countries. Asia shows a prevalence of 28%-32.4%, varying widely among nations. China's prevalence increased from 23.8% to 29%. Few studies exist in Africa, but MAFLD may affect around 20% of the population (Devarbhavi *et al.*, 2023). The total number of deaths among patients with MAFLD has doubled over the last three decades, increasing from 93,757 in 1990 to a total of 168,969 in 2019. Currently, MAFLD is the second-leading cause of liver transplantation in both the US (accounting for 21% of liver transplants in 2018) and Europe (representing 8.4% of liver transplants in 2016). Notably, it is the leading etiology for liver transplantation among females (Adam *et al.*, 2018).

1.4 EXPERIMENTAL ANIMAL MODELS

Many Experimental animal and cell culture models have been established to investigate ALD. These animal models illustrate how alcohol consumption synergistically contributes to lipid dysregulation, oxidative stress, inflammation, and fibrogenesis. The development of ALD in these experimental models depends on various factors, including the composition of an obesogenic diet, alcohol consumption patterns, dosage of alcohol, and genetic background (Buyco *et al.*, 2021). Some animal species commonly used in experimental studies of alcoholic liver disease include rodents (mice and rats), zebrafish, swine, rhesus monkeys, guinea pigs, rabbits, cats, and dogs (Lamas-Paz *et al.*, 2018). In this study, we experimented using zebrafish as our animal model system.

CHAPTER 2

LITERATURE SURVEY

2.1 ZEBRAFISH AN ANIMAL MODEL FOR LIVER DISEASE

The study by (Goessling *et al.*, 2015) highlights the growing significance of zebrafish as a crucial model organism for exploring various aspects of liver biology, including development, regeneration, and diseases. Zebrafish have gained prominence in liver research due to their unique advantages, such as rapid development, transparency of embryos, and genetic tractability. The establishment of the Zebrafish Disease Models Society in 2014 further underscores the increasing interest and investment in utilizing zebrafish to model human liver pathologies.

One notable trend is the broadening scope of hepatobiliary diseases being investigated using zebrafish models. There is an increase in employing zebrafish to study a diverse range of liver diseases, from genetic disorders to environmental toxicities, mirroring the complexity of human liver pathophysiology. Advancements in gene editing technologies have revolutionized zebrafish research, allowing scientists to precisely manipulate the zebrafish genome and create mutants relevant to liver disease. This capability enables the investigation of specific genes involved in liver development, function, and disease progression. Additionally, large-scale chemical screens using zebrafish have become a powerful tool for identifying potential therapeutic compounds. Zebrafish embryos are amenable to high-throughput screening, enabling researchers to test thousands of compounds for their effects on liver health and regeneration.

Furthermore, advanced imaging techniques have greatly enhanced our ability to visualize and analyze hepatic cells in zebrafish. These techniques enable researchers to observe liver development and regeneration processes in real time and track individual cells' behavior over time. Overall, the combination of gene editing technologies, chemical screening methods, and advanced imaging techniques helps zebrafish researchers to contribute to our understanding of liver disease mechanisms significantly. Moreover, the ability to evaluate candidate compounds in zebrafish models provides a valuable platform for preclinical drug

discovery, potentially accelerating the development of novel therapies for liver diseases while minimizing risks associated with clinical trials.

In another similar study conducted by (Lin *et al.*, 2015) a significant breakthrough was achieved in the field of liver disease research by successfully establishing an animal model for ALD using adult zebrafish. Unlike traditional rodent models, the zebrafish model offers a plethora of advantages that enhance its utility in studying ALD. One major advantage is its cost-effectiveness and ease of maintenance. Zebrafish are relatively inexpensive to house and care for compared to larger mammals, making them accessible to a wider range of researchers and institutions. Additionally, their simple husbandry requirements contribute to easier maintenance, allowing for large-scale experiments without significant logistical challenges. Another notable advantage is the absence of surgical expertise requirements. While rodent models often require complex surgical procedures to induce liver injury, the zebrafish model can spontaneously develop liver pathology without the need for invasive interventions. This simplifies experimental procedures and reduces the variability associated with surgical techniques, enhancing the reproducibility of results.

The rapid onset of fibrosis and neoplasia in the zebrafish model is an advantage for studying the progression of ALD. Unlike rodent models, which may take months to develop advanced liver pathology, zebrafish exhibit accelerated disease progression, enabling researchers to observe pathological changes over a shorter timeframe. Despite differences in the anatomical architecture of the zebrafish liver compared to mammals, the study demonstrates that the zebrafish model faithfully recapitulates key pathophysiological features of ALD observed in humans. These include steatosis (fatty liver), steatohepatitis (inflammation and liver cell damage), fibrosis (scarring of liver tissue), and neoplasia (development of liver tumors). This close resemblance to human ALD pathology enhances the translational relevance of findings from zebrafish studies. Overall, this animal model is a valuable tool for advancing our understanding of ALD pathogenesis and associated disorders.

2.2 ETHANOL-INDUCED LIVER INJURY

The authors (Park *et al.*, 2020) of this study have developed a detailed protocol for investigating acute ethanol-induced liver injury using adult zebrafish as a model organism. Their systematic approach to experimentation has yielded important insights into the effects of different ethanol concentrations on liver health in these fish. One key finding is that a higher ethanol concentration of 0.5%, which closely mirrors fatal blood alcohol levels seen in humans, consistently leads to acute liver injury in adult zebrafish when they are exposed to ethanol-containing water for just two days. This rapid onset of liver injury highlights the sensitivity of zebrafish to ethanol exposure and the potential of this model for studying the acute effects of alcohol on the liver.

Notably, it was also observed lethality in zebrafish exposed to ethanol concentrations exceeding 1%, emphasizing the importance of precise dosage considerations in experimental design. By identifying 0.5% ethanol as the optimal concentration for reliably inducing acute liver injury in adult zebrafish, the authors have established a crucial parameter for future research in this area. This standardized protocol enhances the reproducibility of experimental results and provides a foundation for comparing findings across different studies. By elucidating the optimal ethanol concentration and duration of exposure, the authors pave the way for more accurate and reliable investigations into the mechanisms underlying alcohol-related liver damage and the development of potential therapies. Another study was conducted by (Park *et al.*, 2019) to assess the effects of chronic ethanol exposure on adult zebrafish and whether it induces metabolic and pathological features akin to alcoholic liver disease (ALD). This investigation revealed that exposing adult zebrafish to a low dose of ethanol (0.2% ethanol concentration) over a period of 4 weeks resulted in significant elevations in serum glucose and triacylglycerol levels, indicative of disturbances in carbohydrate and lipid metabolism resembling those observed in ALD. Furthermore, an increase in serum alanine aminotransferase (ALT) activity was observed suggesting liver injury following ethanol treatment. This finding aligns with the characteristic hepatocellular damage associated with ALD. At the molecular level, the study also identified the upregulation of genes involved in glucose and lipid metabolism, as well as

mitochondrial biogenesis and function, further substantiating the metabolic alterations induced by ethanol exposure. A heightened expression of genes associated with oxidative and endoplasmic reticulum (ER) stress in the livers of adult zebrafish exposed to chronic ethanol was found. These stress signaling pathways are known to play vital roles in the pathogenesis of ALD, contributing to hepatocyte injury and inflammation.

The study demonstrated by (Passeri *et al.*, 2009) that continuous exposure of zebrafish larvae to 2% ethanol (EtOH) for 32 hours induces characteristic features of acute alcoholic liver disease (ALD), including hepatomegaly, steatosis, and alterations in hepatic gene expression indicative of alcohol metabolism and oxidative stress. By subjecting zebrafish larvae at 4 days post-fertilization (dpf) to 2% ethanol for 32 hours, intracellular ethanol levels reach approximately 80 mM, accompanied by the upregulation of hepatic *cyp2e1*, *sod*, and *bip* genes, suggesting ethanol metabolism and the initiation of oxidative stress. The study further investigated the importance of sterol regulatory element-binding proteins (SREBPs) in acute ALD. Activation of SREBPs is inhibited in *gonzo* larvae, which carry a mutation in the membrane-bound transcription factor protease 1 (*mbtps1*) gene, as well as in embryos injected with a morpholino to knock down SREBP cleavage-activating protein (*scap*). Both *gonzo* mutants and *scap* morphants demonstrate resistance to steatosis in response to 2% ethanol, and the expression of numerous SREBP target genes is downregulated in *gonzo* mutant livers.

This study by (Ellis *et al.*, 2017) entails a methodical approach to studying the acute effects of ethanol exposure on zebrafish larvae. It emphasizes key considerations regarding the timing of ethanol treatment initiation and dosage administration to ensure accurate and reproducible results. The protocol specifies that ethanol treatment should commence no earlier than 96 hours post-fertilization, aligning with the stage at which zebrafish larvae begin to express alcohol-metabolizing enzymes in their liver. This timing is crucial as it ensures that the larvae's metabolic machinery is sufficiently developed to process ethanol, thus reflecting a physiologically relevant experimental model. The maximal tolerated dose of ethanol for zebrafish larvae was identified as 2% by (Passeri *et al.*, 2009). This dosage limit, as reported, is essential for preventing adverse effects such as

high mortality rates or developmental abnormalities. The hepatic steatosis becomes apparent as early as 8 hours after the onset of continuous ethanol exposure, with severity increasing over 24 hours. These findings underscore the dynamic nature of ethanol-induced liver pathology and emphasize the importance of precise timing and dosage control in elucidating its effects on zebrafish larvae.

2.3 HEPATOPROTECTIVE ACTIVITIES

In the study conducted by (Xiong *et al.*, 2019) the molecular mechanisms underlying the antioxidant and anti-inflammatory effects of *Salvia plebeia* R. Br (SPEE) a traditional Chinese medicinal herb, were analyzed in zebrafish through a series of bioassays, it was confirmed that SPEE demonstrated notably stronger inhibition of antioxidant enzyme activities and inflammatory gene expression in zebrafish embryos compared to other tested substances. Furthermore, in adult zebrafish models with thioacetamide-induced liver injuries, SPEE exhibited significant activation of lipid metabolism and redox processes, while simultaneously dampening inflammatory responses. This dual action suggests a multifaceted hepatoprotective role for SPEE in mitigating liver damage induced by toxic insults. Detailed investigations into the anti-inflammatory mechanisms revealed that SPEE influenced cell apoptosis and modulated the expression of pro-inflammatory mediators such as TNF- α , IL6, and IFN- γ . This modulation occurred through the regulation of two key signaling pathways: NF-kB and Nrf2. By modulating these pathways, SPEE exerted its anti-inflammatory effects, thus attenuating the inflammatory cascade associated with liver injury. In conclusion, the findings shed new light on the hepatoprotective properties of SPEE in zebrafish models. The study suggests that SPEE could be a promising candidate for combating inflammation and potentially offers validation for the traditional use of *S. plebeia* in treating inflammatory diseases. Overall, this research underscores the therapeutic potential of SPEE as a natural remedy for inflammatory conditions, offering prospects for the development of novel therapeutic interventions.

The study by (Dai *et al.*, 2022) emphasizes the potential of Magnesium Isoglycyrrhizinate (MgIG) as a therapeutic agent for alleviating acute alcohol-induced hepatic steatosis in zebrafish larvae. The study demonstrated that pretreatment with MgIG led to a significant reduction in acute alcohol-induced ER

stress and dysfunction in lipid metabolism, key mechanisms underlying the development of hepatic steatosis. These findings highlight the effect of MglG in protecting against acute alcohol-induced steatosis by targeting ER stress and lipid metabolism pathways simultaneously. While the study provides promising evidence of MglG's efficacy in ameliorating hepatic steatosis, further investigation is necessary to fully elucidate the precise molecular mechanisms responsible for its hepatoprotective effects in ALD. Moreover, the study introduces and validates a larval zebrafish model of hepatic steatosis induced by alcohol, offering a valuable platform for assessing the effectiveness of anti-steatosis drugs in vivo. This model not only allows for the evaluation of MglG but also serves as a tool for studying the pathogenesis of ALD and screening potential therapeutic interventions.

2.4 CYCLO(-Pro-Tyr)

A study done by (Karanam *et al.*, 2020) focused on a compound known as cyclo(-Pro-Tyr) which is sourced from the bacterium *Bacillus pumilus* AMK1, associated with the marine sponge *Callyspongia fistularis*. The study unveils the anticancer potential of cyclo(-Pro-Tyr), particularly against liver cancer cells like HepG2, highlighting its ability to induce apoptosis or programmed cell death. This apoptosis is largely due to the compound's action on inhibiting the PI3K/AKT signaling pathway, a key driver in cell survival and proliferation often linked to various cancers, including hepatocellular carcinoma. Yet, the investigation is only in its preliminary phase. So far, the effectiveness and safety of cyclo(-Pro-Tyr) have been tested only in cell-based models. Further research in experimental in vivo models is crucial to confirm these results and assess the compound's behavior in more complex biological systems. These steps are critical before progressing to clinical trials involving human participants. In another study conducted by (Karanam *et al.*, 2020) It was demonstrated that cyclo(-Pro-Tyr) notably inhibited cell growth and induced apoptosis in liver cancer cells, an essential mechanism for cancer treatment that involves programmed cell death. A key observation from the research is that cyclo(-Pro-Tyr) induces G2/M phase arrest within the cell cycle. The G2/M phase is a critical juncture where cells ready themselves for division. Arresting the cell cycle at this phase prevents cancer cells from dividing and proliferating, which is a vital strategy in combating cancer growth. These results

highlight the therapeutic potential of naturally derived cyclo(-Pro-Tyr) from *B. pumilus* AMK1. The compound's ability to disrupt the cell cycle and initiate cell death underscores its potential as an effective treatment option for hepatocellular carcinoma.

CHAPTER 3

AIM AND OBJECTIVES

3.1 Aim

- To evaluate the hepatoprotective role of Cyclo(-pro-tyr) against alcohol-associated liver injury in the zebrafish model.

3.2 OBJECTIVES

- Maintenance of the experimental animal model, Zebrafish (*Danio rerio*).
- Induction of liver injury using alcohol model treatment of a group of Zebrafish with or without alcohol.
- To check the hepatoprotective role of cyclo(-pro-tyr) by histopathological and biochemical analysis of antioxidant enzymes.
- RNA isolation and study of inflammatory gene expression using qRT-PCR analysis.

CHAPTER 4

MATERIALS AND METHODS

4.1 MAINTENANCE OF ZEBRAFISH AND EXPERIMENTAL MODEL

The zebrafish were bought from the Sridhar Animal Research Laboratory and Aquarium, Chennai, after getting ethical clearance through the mentor. The zebrafish used for this study was wild-type and 4-month-old male. It was maintained in fresh and clean water with an air pump and a timely food supply (Optimum, Highly Nutritious micro pellet food, rich in vitamins C and E, purchased from southern India Aqua Tek, Chennai). The Zebrafish were then divided into three groups of 10 Zebrafish in three separate one-gallon tanks for the experimental model. All the experimentation was performed in accordance with the institutional animal ethical committee approval no. **SU/CLATR/IAEC/XXII/29/2024**

1. Control
2. Ethanol treatment
3. Cyclo(-pro-tyr) treatment

4.1.1 Control

10 random Zebrafish were selected and maintained in a tank of 1L water for the control group. Water was exchanged once every 24 hours. The Zebrafish were fed twice, morning and evening.

4.1.2 Ethanol treatment

10 random Zebrafish were selected and maintained in a tank of 1L water for the induced group. The induction was done with 0.5% ethanol administered [based on previous reports by (Park *et al.*, 2020). Water with ethanol was exchanged once every 24 hours. The Zebrafish were fed twice, morning and evening.

4.1.3 Cyclo(-pro-tyr) treatment

Another 10 random Zebrafish were selected for the cyclo(-pro-tyr) treatment and maintained in a tank of 1L water. Cyclo(-pro-tyr) was dissolved in water

(91mg/1000ml)–(350 μ M) with 0.5% ethanol. Water with ethanol and cyclo(-pro-tyr) was exchanged once every 24 hours. The Zebrafish were fed twice in the morning and evening.

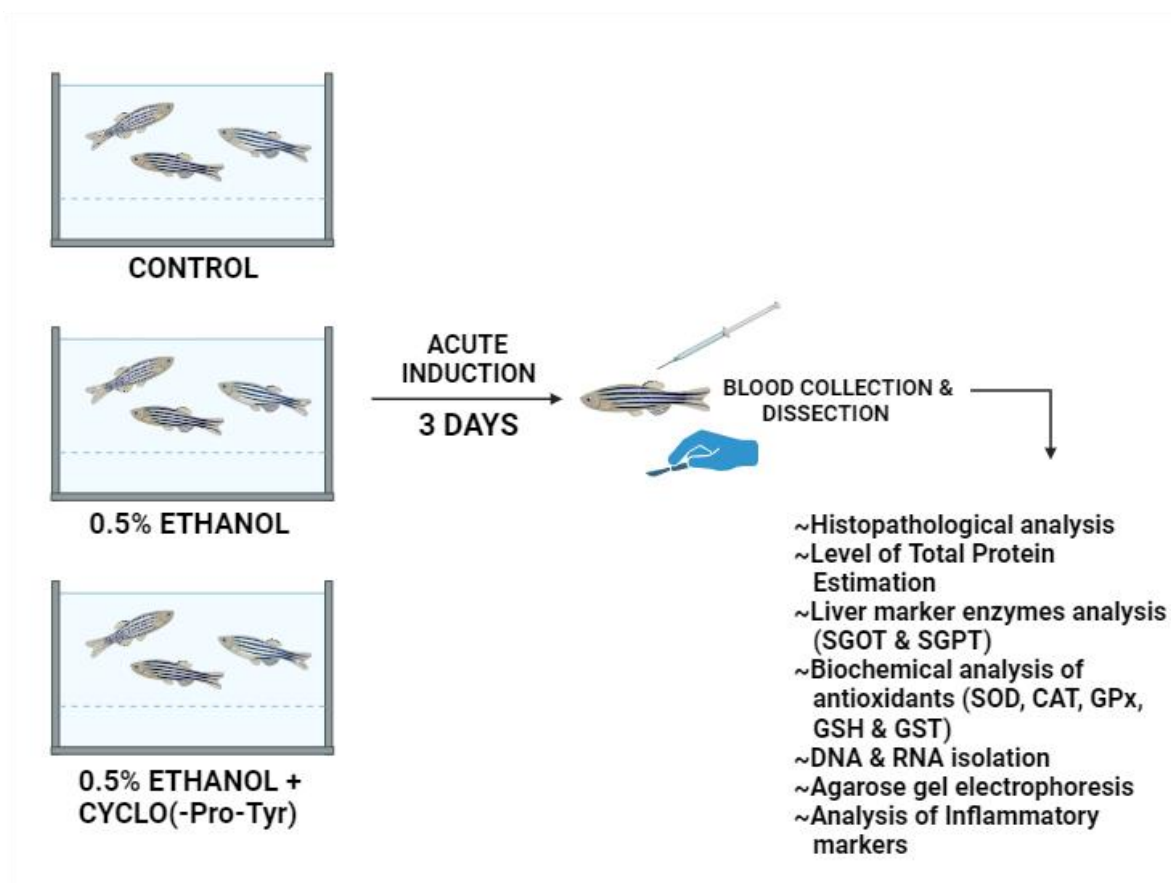


Fig: 4.1: Experimental design

4.2 BLOOD COLLECTION AND DISSECTION

After 3 days of ethanol treatment and cyclo(-pro-tyr) treatment. Blood collection and dissection were performed based on previous reports (Zang *et al.*, 2015) and (Gupta *et al.*, 2010)

4.2.1 Blood collection

Materials required

- Insulin Syringe
- Ethylenediamine tetraacetic acid (EDTA)

- Phosphate buffer saline (PBS)
- Na_2HPO_4 (Sodium hydrogen phosphate)
- NaH_2PO_4 (Sodium dihydrogen phosphate)
- NaCl (Sodium chloride)
- Distilled water

Procedure

As per the standard protocol, PBS was prepared by adding Na_2HPO_4 , NaH_2PO_4 and NaCl to the distilled water. Then EDTA was prepared by dissolving in PBS. Then a Zebrafish from the control group was taken, pat-dried using a paper towel, and then it was placed on the table for blood collection. Then, a small amount of EDTA was taken in the insulin syringe. The syringe needle was placed near the zebrafish's caudal fin, EDTA was injected, and then the syringe's plunger was pulled out slowly so the blood rose in the syringe. This was done for three Zebrafish, one Zebrafish from each group. The collected blood samples were transferred to the respectively labeled Eppendorf. After that centrifugation was done and the serum supernatant was collected. The collected serum supernatants were diluted at 1:10 in PBS and were stored at -20°C .



Fig 4.2 Blood collection using insulin syringe

4.2.2 Dissection

Materials required

- Surgical blade
- Trizol
- 10% formaldehyde
- PBS

Procedure

The PBS was prepared as mentioned earlier. Then 10% formaldehyde was prepared with PBS. A Zebrafish from the control was taken out, pat-dried on a paper towel, and placed on the table for the dissection. The skin of the Zebrafish was snipped using a surgical blade. Then the liver was removed carefully. The removed liver was transferred to a labeled eppendorf with 500 μ l PBS. After removing the liver, the remaining whole tissue was cut into a few pieces and then added to a separately labeled eppendorf with 500 μ l PBS. The above procedure was repeated for the other two Zebrafish, one Zebrafish each from ethanol and cyclo(-pro-tyr) groups. Three liver samples and three whole tissue samples were then homogenized using a homogenizer. The homogenized sample was then centrifuged and the supernatant was collected. It was stored at -20°C. Another three Zebrafish were taken, one Zebrafish from each group. The Zebrafish's caudal fin was removed. Then these Zebrafish were added to a falcon tube with 10% formaldehyde.

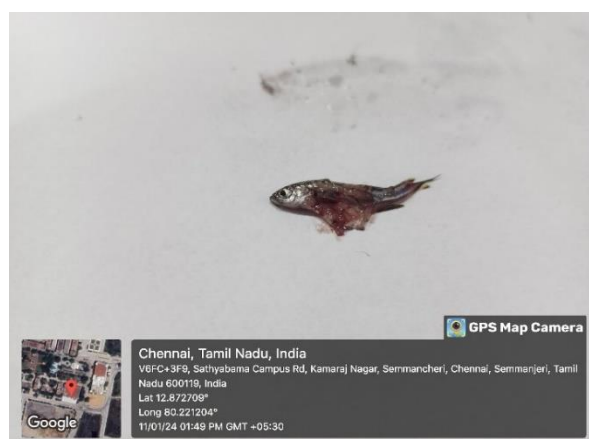


Fig 4.3 Dissection of zebrafish

4.3 HISTOPATHOLOGICAL ANALYSIS

Histopathology involves the diagnosis and study of diseases of the tissues by examining tissue and/or cell alterations. This method is useful to detect potentially deleterious effects of compounds on a particular organ or tissue. Observed morphological alterations in the gills, liver, and kidneys of individual Zebrafish were recorded in a table of histological examination. Several organs of zebrafish have been used in toxicological studies, such as the gonads, pharynx, thyroid, intestine, liver, kidney, gills, and muscles. A considerable advantage of zebrafish in histopathology is the possibility to observe several organs in only one slide due to their small size. As for the liver, there are relevant similarities between the liver of zebrafish and those of superior mammals in the most basal aspects and regulatory mechanisms related to hepatotoxicity (Custódio de Souza *et al.*, 2019).

The Zebrafish samples that were added to the 10% formaldehyde were given for histopathology by Hematoxylin & Eosin (H&E) staining at Sathyabama Dental College and Hospital, Chennai. The received slides were observed under the light microscope for tissue damage and morphological changes in the liver.

4.4 TOTAL PROTEIN ESTIMATION

The unknown concentration of the protein in the sample is determined using Lowry's method (Lowry *et al.*, 1951). Lowry's assay for total protein estimation is one of the most commonly used colorimetric assays. Protein content in the liver and whole tissues were calculated based on the protocol (Waterborg *et al.*, 2009).

The total protein can be estimated based on two reactions that take place. The first reaction is the reduction of copper ions under alkaline conditions, which forms a complex with peptide bonds. The second reaction is the reduction of the Folin-Ciocalteu reagent by the copper-peptide bond complex, which subsequently causes a color change of the solution into blue with an absorption in the range of 570 to 650nm detectable with a spectrophotometer.

Reagents required

- Standard bovine serum albumin (BSA) (Concentration 100µg/ml)
- Reagent 1: Alkaline copper sulphate reagent
 - Sol A: 2% Na₂CO₃ in 0.1N NaOH (Sodium hydroxide)
 - Sol B: 0.5% CuSO₄ in H₂O
 - Sol C: 1% KNaC₄H₄O₆ (Sodium potassium tartrate) in H₂O.
 - 48ml of Sol A + 1ml of Sol B + 1ml of Sol C
 - Prepare freshly before use.
- Reagent 2: Folin Phenol Reagent
 - 1 part Folin-Phenol [2 N]: 1 part water

Procedure

In a 96-well plate, 20µl of BSA was added in duplicates for the standard. Then 10µl of samples were added in duplicates. For the blank well neither BSA nor the samples were added. Distilled water was then added to equal the volumes of all the wells. After that reagent 1 was added to all the wells. Then, incubation was done for 10 minutes. After the incubation reagent 2 was added to all the wells. It was then incubated for 30 minutes. Then the OD value was taken at 570nm in the multi-plate reader EPOCH, Biotek, Agilent.

4.4.1 Total protein concentration

The total protein concentration was calculated using the formula given below

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

4.5 LIVER MARKER ENZYMES ANALYSES

Elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme levels in the blood are linked to a higher risk of illness and mortality from all causes. They are also known as serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) respectively. These enzymes are key indicators of liver health, with their levels serving as predictors for the presence of liver diseases. The measurement of ALT and AST levels is a standard method to gauge liver damage. An increase in these enzymes typically signals damage to liver cells, highlighting the occurrence of hepatocellular damage (van Beek *et al.*, 2013).

SGOT and SGPT were analyzed following the method of (Karanam *et al.*, 2022) in the blood and liver samples of the three experimental groups.

4.5.1 SGOT/AST

SGOT or AST, is an enzyme that catalyzes the reversible reaction of transferring an amino group from aspartate to α -ketoglutarate, then forming glutamate and oxaloacetate. The principle involves measuring the rate of this reaction. The activity of SGOT is estimated by observing the amount of oxaloacetate produced, then it is reacted with 2,4-Dinitrophenylhydrazine to form a hydrazone. This hydrazone can be measured in a spectrophotometer at a wavelength of 540nm, it is proportional to the enzyme activity in the sample.

Materials required

- Buffer substrate (Aspartic acid)
- α KG (α -ketoglutaric acid)
- 0.1M PO_4 (phosphate) buffer, pH 7.4
- DNPH (2,4-Dinitrophenylhydrazine) prepared in 1N HCl (Hydrochloric acid)
- 0.4M NaOH (Sodium hydroxide)

Procedure

As per the standard protocol, the aspartic acid buffer substrate was prepared with aspartate, α KG and 0.1M PO_4 buffer. Then the 96-well plate was placed on the ice and buffer substrate was added to all the required wells. 20 μ l of samples were added to the 96-well plate in triplicates. The blank well had no sample. Following that, 50 μ l of DNPH was added. The plate was covered with a foil and incubated in the dark for 20 minutes. Then 40 μ l of 0.4M NaOH was added to all the wells. The reading was taken at 540nm in the multi-plate reader EPOCH, Biotek, Agilent.

4.5.1.1 Activity of SGOT

The activity of SGOT was calculated using the given formula

$$= \frac{\text{Test OD}}{0.01} \times 10 \times \frac{1}{60}$$

and was normalised to mg of tissue protein.

4.5.2 SGPT/ALT

SGPT/ALT enzyme also has a similar function of catalyzing the transfer of an amino group, but from alanine to α -ketoglutarate, forming glutamate and pyruvate. The principle involves measuring the rate of this transamination reaction. Pyruvate is measured by its reaction with 2,4-Dinitrophenylhydrazine in the spectrophotometer at 540nm.

Materials required

- Buffer substrate (Alanine)
- α KG (α -ketoglutaric acid)
- 0.1M PO_4 (phosphate) buffer, pH 7.4
- DNPH (2,4-Dinitrophenylhydrazine) prepared in 1N HCl (Hydrochloric acid)
- 0.4M Sodium hydroxide (NaOH)

Procedure

The alanine buffer substrate was prepared with alanine, α KG and 0.1M PO_4 buffer. Then the prepared alanine buffer substrate was added to all the wells while placing the 96-well plate on the ice. 20 μ l of samples were added in triplicates. No sample was added to the blank well. After that, 50 μ l of DNPH was added. The plate was covered using foil and incubated in the dark for 20 minutes. 40 μ l of 0.4M NaOH was added to all the wells. Finally, the reading was taken at 540nm in the multi-plate reader EPOCH, Biotek, Agilent.

4.5.2.1 Activity of SGPT

The activity of SGPT was calculated using the given formula

$$= \frac{\text{Test OD}}{0.01} \times 10 \times \frac{1}{60}$$

and was normalised to mg of tissue protein.

4.6 BIOCHEMICAL ANALYSIS FOR ANTIOXIDANTS

Biochemical analysis techniques are a diverse set of methods used to analyze biochemical substances in living organisms, revealing essential chemical reactions. These methods detect and quantify biomolecules such as enzymes, carbohydrates, lipids, and proteins, along with other biochemical parameters like inorganic elements and liver function indicators. It plays an important role in the diagnosis, monitoring, and treatment of a range of medical conditions.

Biochemical analyses of liver and whole tissue samples of the three groups of Zebrafish were performed based on the previous reports. This was done to analyze and compare the effect of cyclo(-pro-tyr) on the antioxidant enzyme activity of Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxide (GPx), Glutathione (GSH) and Glutathione S-transferase (GST) of the three experimental groups.

4.6.1 SOD

SOD is an important antioxidant enzyme that catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. The pyrogallol autoxidation method by (Marklund *et al.*, 1974) was used to measure SOD. In this method, pyrogallol autoxidizes in the presence of molecular oxygen to produce superoxide anion. The presence of SOD in the sample inhibits the oxidation of pyrogallol by scavenging the superoxide anions, this reduces the rate of pyrogallol autoxidation. The decrease in the pyrogallol autoxidation is measured in the spectrophotometer at 470nm.

Materials required

- Absolute ethanol
- Chloroform
- 0.1M Tris buffer, pH 8.2
- Distilled water
- Pyrogallol (1,2,3-trihydroxybenzene)

Procedure

In a 96-well plate, samples were added in triplicates. The well with no sample acts as blank. Then the absolute ethanol was added to all the wells. Following that chloroform was added. It was then incubated for 15 min. After the incubation, 0.1M tris buffer was added. Distilled water was added next. And lastly, pyrogallol was added just before taking the reading. Then the OD value was taken at 470nm in the multi-plate reader EPOCH, Biotek, Agilent.

4.6.1.1 Activity of SOD

The activity of SOD was calculated with the given formula

$$\frac{OD}{10} \times \frac{vol\ of\ well}{vol\ of\ sample} \times \frac{1}{0.5}$$

and was normalised to mg of tissue protein.

4.6.2 CAT

CAT is an antioxidant enzyme that decomposes hydrogen peroxide into water and oxygen. CAT activity was measured using potassium dichromate according to the method described by (Sinha *et al.*, 1972). In this method potassium dichromate is a strong oxidizing agent, it reacts with hydrogen peroxide and forms chromic acetate. The chromic acetate produced is measured in the spectrophotometer at 590nm. The CAT activity is inversely proportional to the amount of potassium dichromate consumed.

Materials required

- 0.01M PO₄ (phosphate) buffer, pH 7.0
- 0.2M H₂O₂ (Hydrogen peroxide)
- K₂Cr₂O₇ (Potassium dichromate)

Procedure

First, the samples were added as triplicates in the 96-well plates. The sample was not added in the blank well. To all the wells 0.01M PO₄ buffer was added. Then H₂O₂ was added to all the wells. After that KCr₂O₇ was added. The incubation time was 10 minutes. After the incubation, the plate was read at 590nm in the plate reader.

4.6.2.1 Activity of CAT

The activity of CAT was calculated by the given formula

$$\frac{\text{Test OD}}{\text{Blank OD}} \times 3.4 \times \frac{1000}{\text{vol of sample}} \times 10$$

and was normalised to mg of tissue protein.

4.6.3 GPx

GPx is an antioxidant enzyme that catalyzes the reduction of hydrogen peroxide to oxidized glutathione using reduced glutathione as a cofactor. GPx activity was measured using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), a yellow coloured compound based on previously described by (Rotruck *et al.*, 1973). In this method, the oxidized glutathione produced reacts with DTNB and forms a yellow-coloured complex. The absorbance of the yellow-coloured complex is measured spectrophotometrically at 412nm.

Materials required

- 0.04M PO₄ (phosphate) buffer, pH 7.0
- 10mM NaN₃ (Sodium azide)
- 4mM Reduced GSH (glutathione)
- 2.5mM H₂O₂ (Hydrogen peroxide)
- 10% TCA (trichloroacetic acid)
- 0.3M PO₄ (phosphate) solution
- DTNB (5,5'-dithiobis (2-nitrobenzoic acid))

Procedure

To the 96-well plate, the samples were added as triplicates. For the blank, no sample was added. Then 0.04M PO₄ buffer was added to all the wells. 10 mM NaN₃ was added after that. Next, to all the wells 4mM Reduced was added. Then 2.5 mM H₂O₂ was added. After that, to all the wells distilled water was added. It was incubated for 3 minutes. Followed by, 0.3M PO₄ solution was added. Then DTNB was added to all the wells. 10% TCA was added to only the sample containing wells. The readings were taken at 412nm in the multi-plate reader EPOCH, Biotek, Agilent.

4.6.3.1 Activity of GPX

The activity of GPX was calculated by the following formula

$$\frac{\text{Test OD}}{0.11} \times 10 \times \frac{2}{\text{vol of sample}} \times 1/0.2$$

and it was normalized to mg of protein.

4.6.4 GSH

GSH is an antioxidant maintained in reduced form. Reduced GSH gets converted to oxidized glutathione upon oxidation. GSH activity was measured using the protocol prescribed by (Ellman *et al.*, 1959). In this method, GSH reacts with DTNB and forms a yellow-coloured compound. The compound formed is measured in a spectrophotometer at 412nm.

Materials required

- 10% TCA (trichloroacetic acid)
- 0.3M PO₄ (phosphate) solution
- DTNB (5,5'-dithiobis (2-nitrobenzoic acid))
- Distilled water

Procedure

The samples were added in triplicates in the 96-well plate. No sample was added to the blank well. Then, 10% TCA was added to all the wells. 0.3M PO₄ was added next. After that DTNB was added. Then the multi-plate was incubated for 10 minutes. The plate was read at 412nm in the multi-plate reader EPOCH, Biotek, Agilent.

4.6.4.1 Activity of GSH

The activity of GSH was calculated by the following formula

$$\frac{\text{Test OD}}{0.11} \times 10 \times \frac{2}{\text{vol of sample}} \times 1/0.2$$

and it was normalized to mg of protein.

4.6.5 GST

GST is a GSH-mediated antioxidant involved in the detoxification of harmful compounds. GST catalyzes the transfer of GSH to electrophilic substrates by forming a conjugate between GSH and the substrate. GST activity was measured based on previous research (Habig *et al.*,1974). In this method, 1-Chloro-2,4-dinitrobenzene (CDNB) is commonly used. The CDNB reacts with GSH to form a conjugate. The formed conjugate can be detected by measuring in a spectrophotometer at 340nm.

Materials required

- 0.5M PO₄ (Phosphate) buffer, pH 6.5
- 25mM CDNB (1-Chloro-2,4-dinitrobenzene)
 - Prepared in 95% ethanol
- Distilled water
- 20mM Reduced GSH (Glutathione)

Procedure

To the 96-well plate, samples were added in triplicates. The well with no sample acts as a blank. After that CDNB was added. Then, 0.5M PO₄ was added to all the wells including blank. The plate was incubated for 5 minutes. Lastly, 20mM

reduced GSH was added to all the wells. The plate was read at 340nm in the plate reader.

4.6.5.1 Activity of GST

The activity of GST was calculated by the following formula

$$\frac{\text{Test OD}}{0.11} \times 10 \times \frac{2}{\text{vol of sample}} \times \frac{1}{0.2}$$

and it was normalized to mg of protein.

4.7 DNA ISOLATION

DNA isolation was performed for the whole tissue of the three experimental groups, based on the previous research (Karanam *et al.*, 2020)

Materials required

- Homogenize buffer, pH 8.0
- 10mM EDTA (Ethylenediamine tetraacetic acid)
- 20% SDS (Sodium dodecyl sulphate)
- Phenol
- Chloroform: Isoamyl alcohol (24:1)
- 4M NaCl (Sodium chloride)
- 0.9% NaCl
- Absolute ethanol
- 60% ethanol
- Milli-Q water

Procedure

As per the standard protocol, the homogenize buffer was prepared with 10 mM EDTA in 0.9% NaCl. Then the homogenize buffer was added to the body tissues of three Zebrafish, one from each group. It was then homogenized and centrifuged at 1000 rpm for 5 minutes. The supernatant was collected in the eppendorf tube. To the collected supernatant 20% SDS, equal amounts of phenol and chloroform: Isoamyl alcohol mixture was added. It was mixed thoroughly and centrifuged at 11,000 rpm for 5 minutes. The aqueous layer was collected carefully and transferred to another tube. 4M NaCl and 100% ethanol were added to the collected aqueous layer. Then it was centrifuged at 12,000 rpm for 5 minutes. The pellet was collected. It was then washed with 60% ethanol twice. After that, it was air-dried. Once air-dried 80-90% Milli-Q water was added. It was stored at -20°C.

4.8 AGAROSE GEL ELECTROPHORESIS

Materials required

- TBE (tris borate EDTA) buffer
- Tris base
- Boric acid
- EDTA (Ethylenediamine tetraacetic acid)
- Deionized water
- 1% Agarose gel
- EtBr (Ethidium Bromide)
- Casting tray and comb
- Electrophoresis unit tank
- Power supply
- Bromophenol blue
- glycerol
- DNA samples

Procedure

First, 10x TBE buffer was prepared for 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 thoroughly boiled 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 in chemidoc, XRS + system, (Biosrad) USA.

4.9 RNA ISOLATION

RNA isolation was performed for the whole tissue of the three experimental groups, based on the protocol (Peterson *et al.*, 2009).

Materials required

- Trizol reagent
- Chilled chloroform
- Chilled isopropanol
- 95% ice chilled ethanol
- RNase-free water

Procedure

The samples of the whole tissue of the three Zebrafish, one Zebrafish each from one group which was already stored in the trizol were taken after the incubation of 48h. Then it was 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 then centrifuged at 8000 rpm for 15 minutes at 4°C. 3 phases were visible, and the colourless upper layer was transferred to another tube. Ice-chilled isopropanol was added and incubated for 15 minutes at room temp. Then 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. The steps from 10 to 12 were repeated. After that, it was allowed to air dry in the hood but not completely. Once air-dried 80-90% RNase-free water was added. It was stored at -20°C.

4.10 ANALYSIS OF INFLAMMATORY MARKERS THROUGH qRT-PCR

The isolated RNA was converted to cDNA and the expression levels of the TNF- α , NF- κ B, IL-1 β and IL-6 and were examined based on the manufacturer's instruction.

Materials required

- Forward and Reverse primers of the target gene's
- qPCR Supermix (64142197)
- cDNA template
- Nuclease free water
- cDNA conversion (#1708891)

Table 4.1: List of primers used for qRT-PCR

S.No	TARGET GENE	GENE ID	FORWARD PRIMER	REVERSE PRIMER	AMPLICON LENGTH (bp)
1.	TNF- α	3553	AACCATTCT CCTTCTCCCC	TTTCACCTCC ATCCATCCTC	186
2.	NF- κ B	4791	CGAATGGA CAAGACAGCAG	CCGGAACAC AATGGCATA	177
3.	IL-1 β	7124	CTCTCACCT CTCCTACTCAC	ACACTGCTA CTTCTTGCC C	194
4.	IL-6	3569	ATAACCACC CCTGACCC	ATACCTCAAA CTCCAAAAG	149

Procedure

First master mix, forward primers, reverse primers and cDNA template were combined for the qRT-PCR setup. The volume was then adjusted with nuclease-free water. PCR amplifications were done using the thermal cycler. 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.

4.10.1 Gene expression identification

The results were expressed as a relative expression of the mRNA gene in fold change to the control, where a fold change of more than 1.0 indicated upregulation of the gene and less than 1.0 indicated downregulation of the gene expression.

4.11 STATISTICAL ANALYSIS

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

CHAPTER 5

RESULTS AND DISCUSSION

5.1 MAINTENANCE OF ZEBRAFISH AND EXPERIMENTAL MODEL

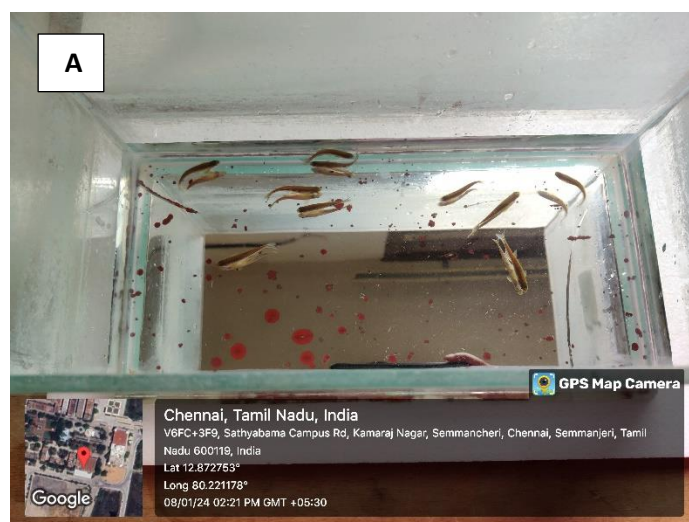


Fig: 5.1: Experimental group design (A) Control (B) Ethanol treatment (C)Cyclo(-pro-tyr) treatment

5.2 HISTOPATHOLOGICAL RESULTS

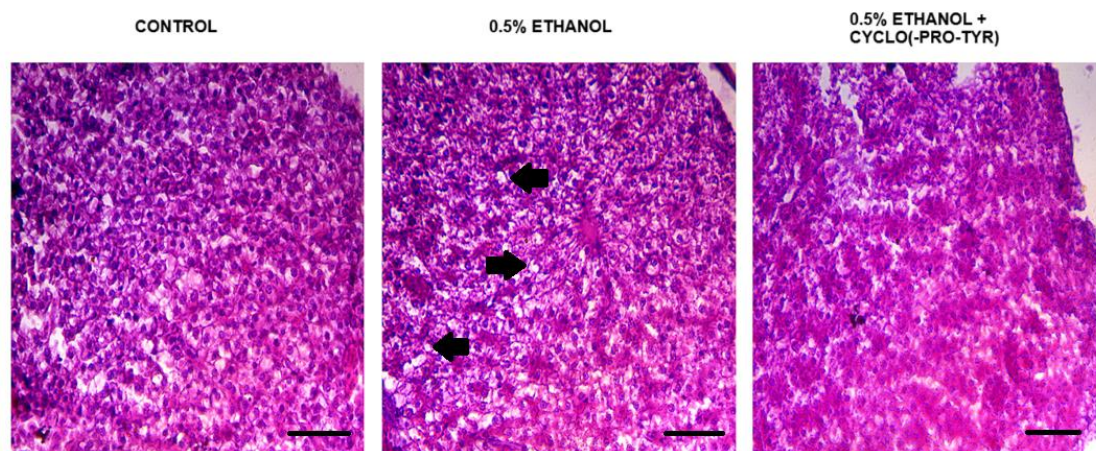


Fig:5.2 : The Hematoxylin and Eosin (H&E) staining of liver sections from zebrafish treated with Cyclo(-pro-tyr) for 3 days. Photomicrograph of representative H&E stained liver sections of control, ethanol, and Cyclo(-pro-tyr)-supplemented in zebrafish. Micro and macrovesicular steatosis was observed in liver sections of ethanol-fed zebrafish, while liver sections of Cyclo(-pro-tyr)-supplemented ethanol Zebrafish liver showed microvesicular steatosis. Data are presented as the mean $\pm$ SEM ($n = 3$); values not sharing a common letter differ significantly from each other at $p \leq 0.05$. Scale bar = 50 μm (Magnification 40X).

The deposition of round droplets in the hepatocytes is indicative of micro steatosis, which is the accumulation of small fat droplets in liver cells in zebrafish treated acutely with 0.5% ethanol and 0.5% ethanol + Cyclo(-pro-tyr). Normal staining patterns, with pink-stained cytoplasm and blue-purple nuclei, were seen in tissue sections free of microsteatosis. Fig 5.2 The hepatocytes with microsteatosis are marked with red marking arrows. (Sampey *et al.*, 2003). When compared to ethanol induction, treated Cyclo(-pro-tyr) exhibits normal morphology.

5.3 TOTAL PROTEIN QUANTIFICATION

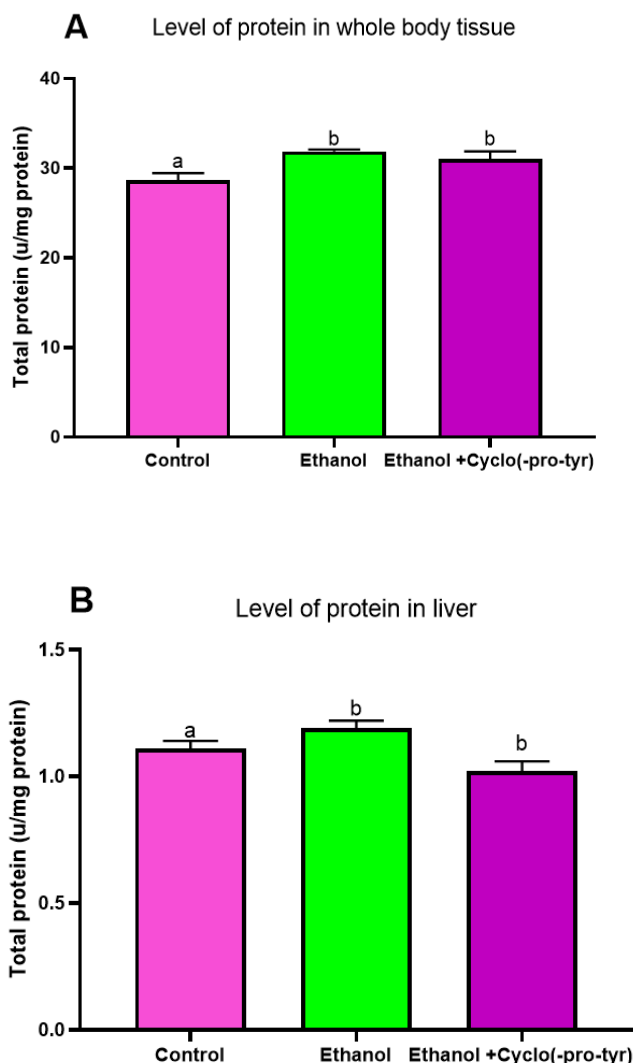


Fig:5.3: Level of total protein (A) in whole body tissue and (B) in liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n=3$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

The amounts of total protein were assessed in three groups: Control, 0.5%Ethanol, and 0.5% Ethanol +350 μ M Cyclo(-pro-tyr). Figure 5.3(A) depicts the total protein levels in whole-body tissue, while Figure 5.3(B) depicts the protein levels in the adult zebrafish's liver. According to Figure 5.3's results, the ethanol group's protein levels were the highest. However, in the liver the protein level was found to be the lowest in ethanol+cyclo(-pro-tyr) group.

5.4 ANALYSES OF LIVER MARKER ENZYMES

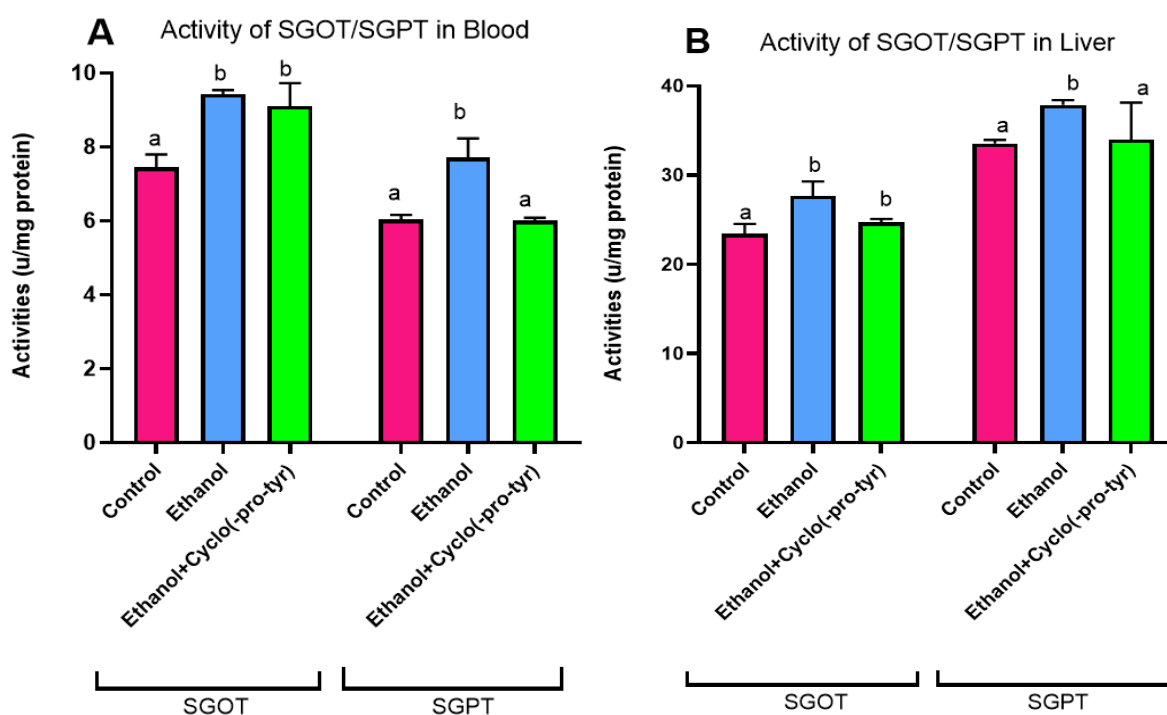


Fig:5.4: Activities of SGOT/SGPT (A) in blood and (B) in liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 3$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

SGOT and SGPT enzymes are distributed across various tissues in the body, with liver tissues containing particularly high concentrations. When liver cells are damaged or destroyed, SGOT and SGPT are released into the bloodstream, serving as valuable indicators of liver damage or disease. In Fig 5.4, the acute induction of ethanol and treatment illustrates the activity of SGOT/SGPT enzymes in both blood and liver. The treated group exhibited a decrease in activity compared to the ethanol group of Zebrafish.

5.5 BIOCHEMICAL ANALYSIS

5.5.1 SOD activity

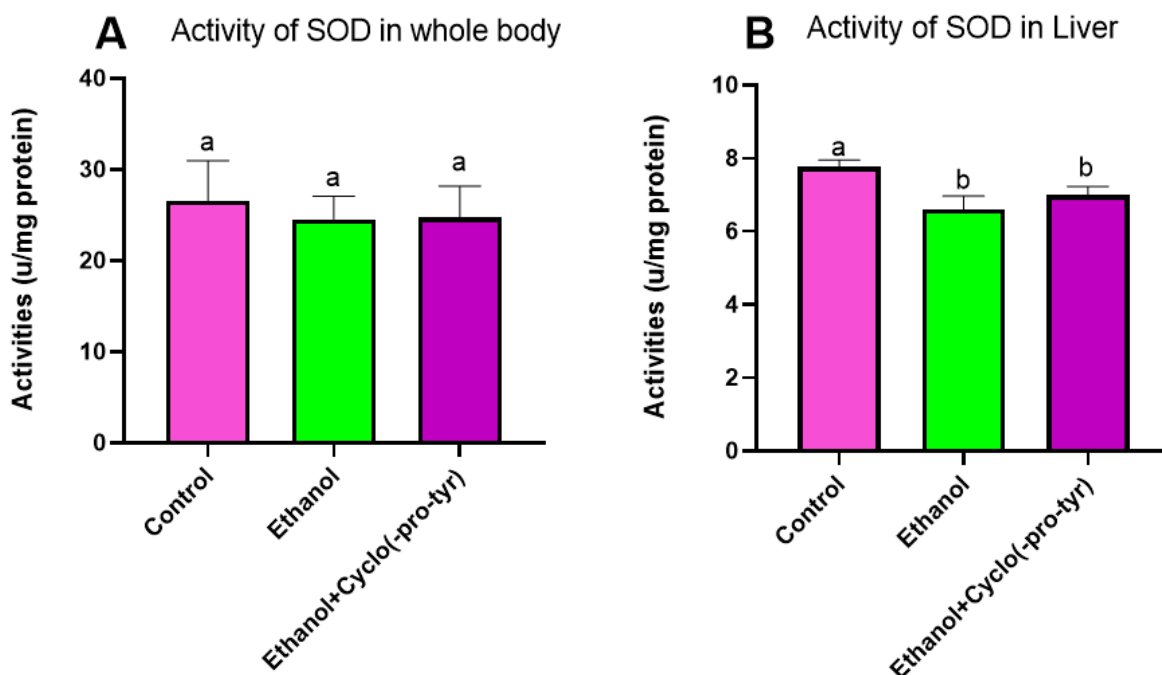


Fig: 5.5: Activity of an enzyme Superoxide (SOD), (A) in whole body tissue and (B) in liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 3$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

Superoxide Dismutase (SOD) enzyme plays a critical role in defending against oxidative stress by converting superoxide radicals into hydrogen peroxide and oxygen. In Fig 5.4, the acute induction of ethanol and treatment illustrates the activity of the SOD enzyme in both blood and liver. The activity of the SOD enzyme is observed to be increased in the treated group compared to the ethanol group.

5.5.2 CAT activity

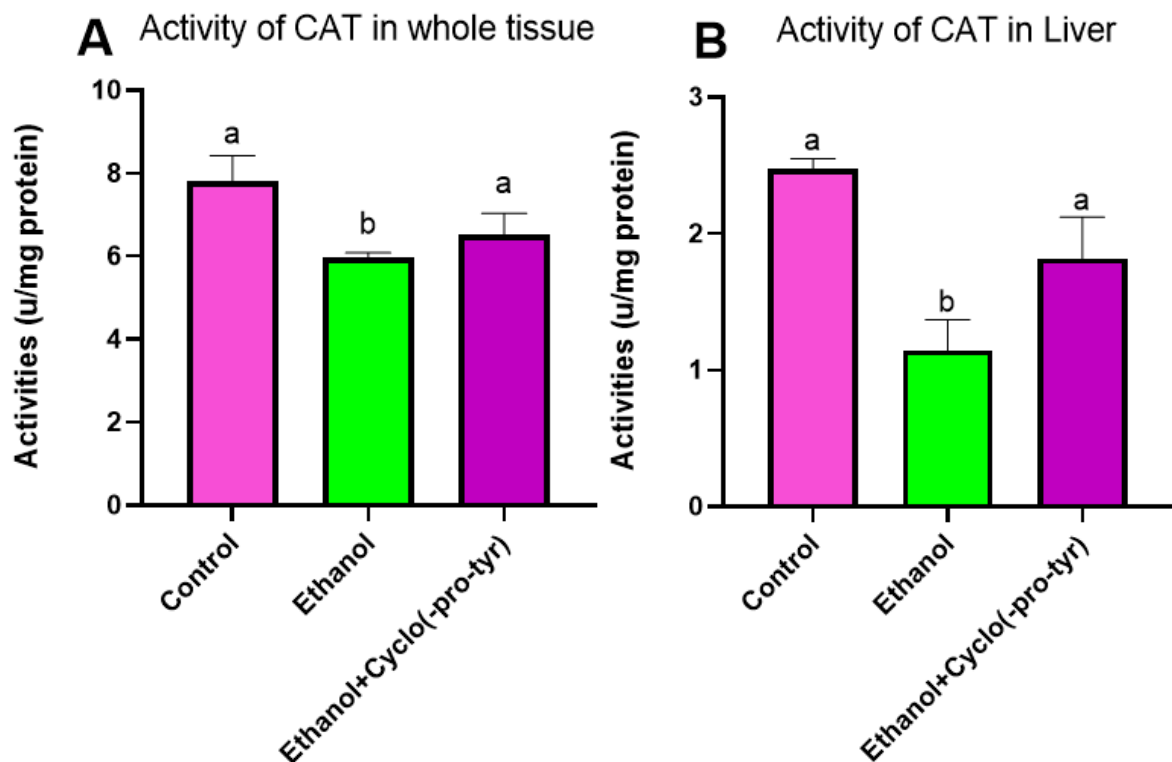


Fig: 5.6: Activity of an enzyme CAT (A) in whole-body tissue and (B) in liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 3$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

CAT is an important enzyme found that helps convert hydrogen peroxide into water and oxygen. This reaction is crucial for the detoxification of hydrogen peroxide, a harmful by-product of various metabolic processes, into harmless substances. In Fig 5.5 the acute induction of ethanol and treated represents the activity of CAT enzyme in whole body tissue and liver. The activity of the CAT enzyme is increased in the treated group compared to the ethanol group.

5.5.3 GPx activity

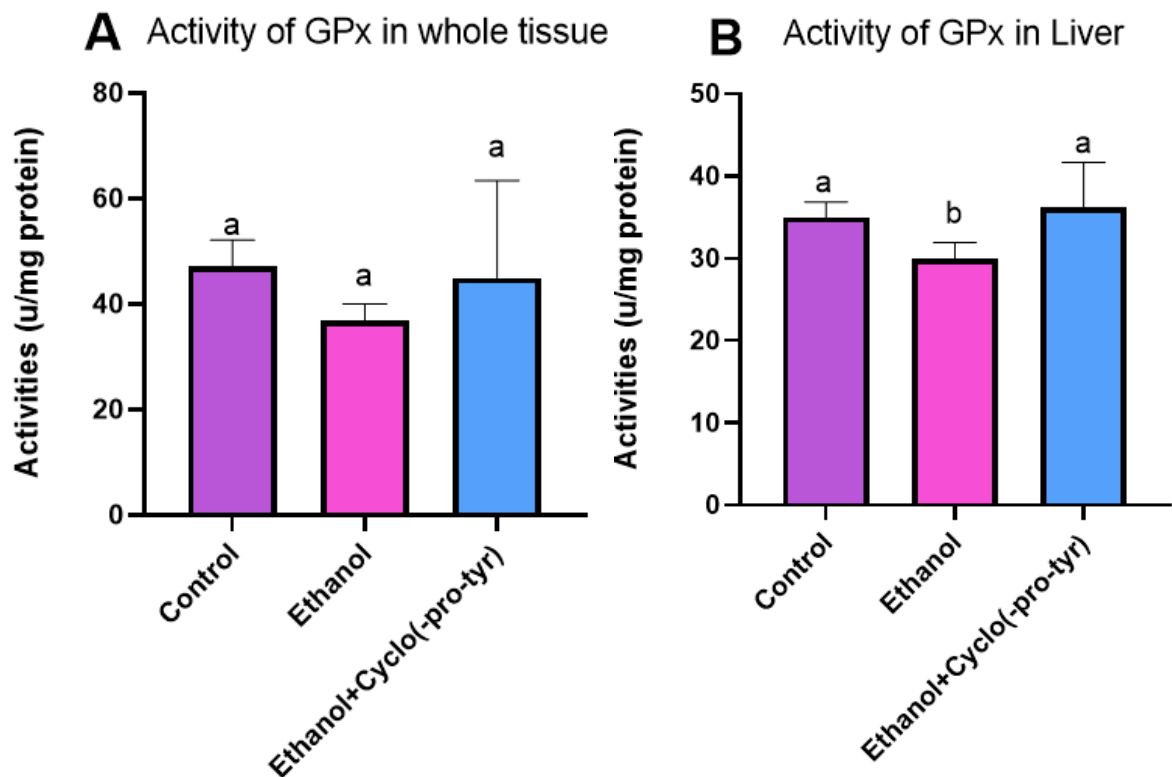


Fig: 5.7 : Activity of an enzyme GPx (A) in whole body tissue and (B) in liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 3$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

In Fig 5.6 the acute induction of ethanol and treated represents the activity of GPx enzyme in whole body tissue and liver. The activity of the GPx enzyme is increased in the treated group compared to the ethanol group. This indicates that the activity gets restored back in the cyclo(-pro-tyr) group.

5.5.4 GSH activity

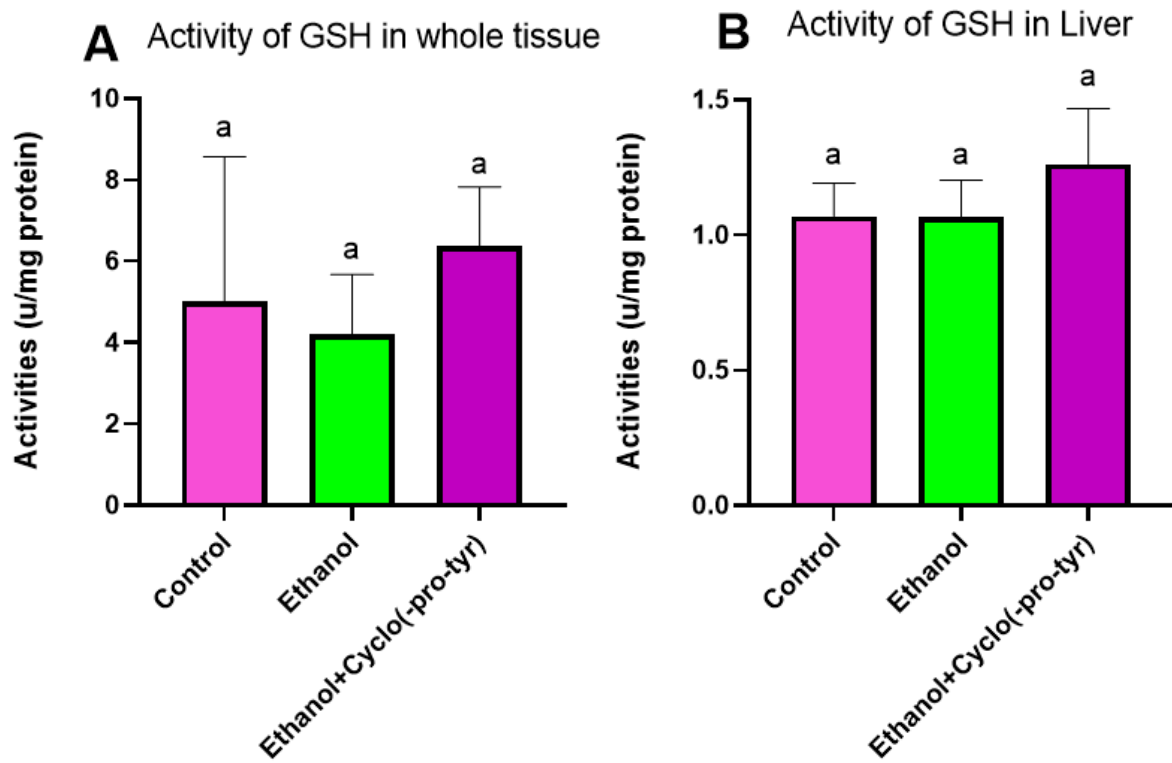


Fig: 5.8: Activity of an enzyme GSH (A) in whole body tissue and (B) in liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 3$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

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 the GSH enzyme is increased in the treated group compared to the ethanol group. Indicating that the activity gets restored back in the cyclo(-pro-tyr) group.

5.5.5 GST activity

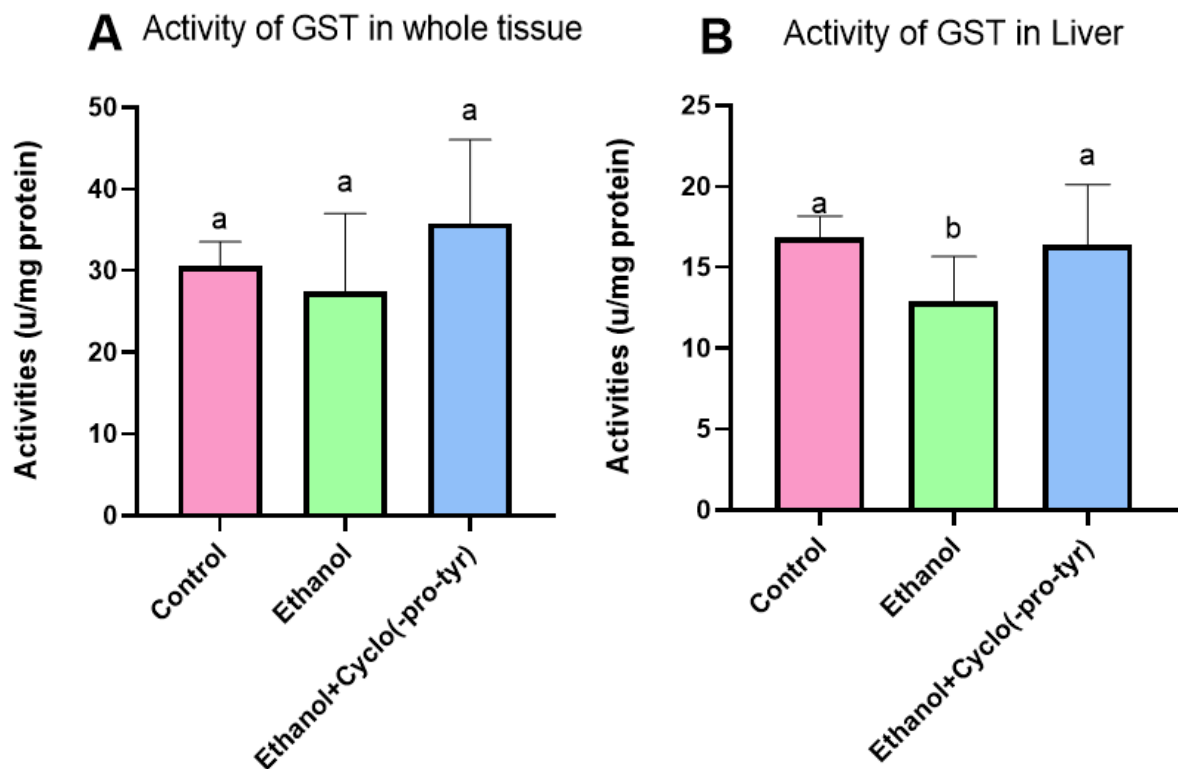
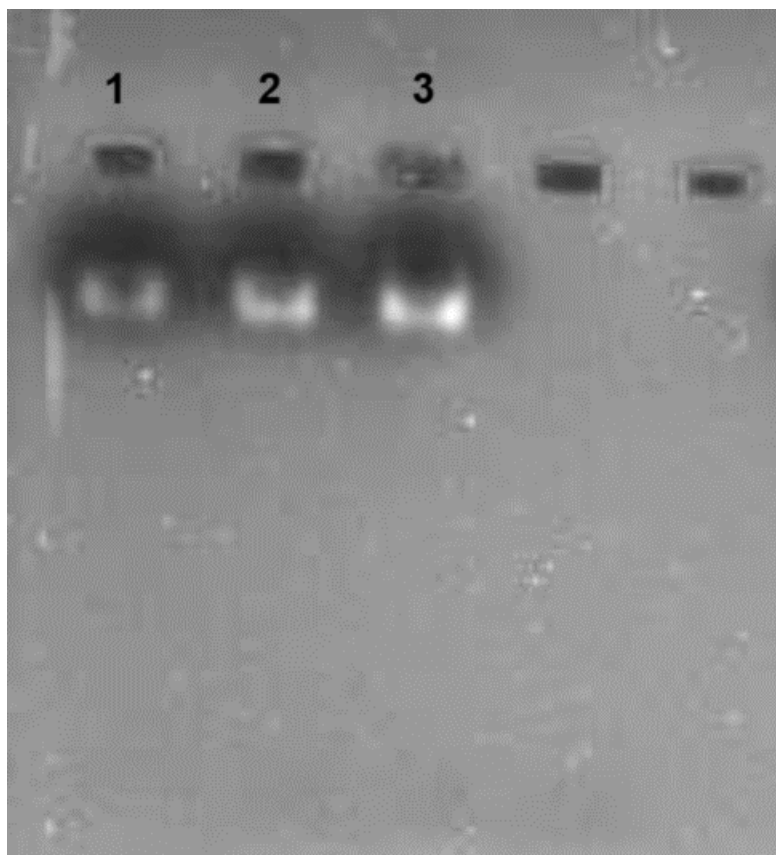


Fig:5.9: Activity of an enzyme GST (A) in whole body tissue and (B) in liver of acute induction of ethanol in zebrafish. Data are presented as the mean $\pm$ SEM ($n = 3$); values not sharing a common letter significantly differ from each other at $p \leq 0.05$.

GST plays a role in detoxification. In Fig 5.8 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 77 increased in treated group compared to ethanol group. This indicates that cyclo(-pro-tyr) helps in restoring back the activity of GST.

5.6 AGAROSE GEL ELECTROPHORESIS



Lane 1: Control

Lane 2: 0.5% Ethanol

Lane 3: 0.5% Ethanol + Cyclo(-pro-tyr)

Fig: 5.10: DNA FRAGMENTATION ANALYSIS

Fragmentation of the DNA was analyzed of the isolated DNA in the experimental group of animals through agarose gel electrophoresis and visualized under UV light. The results showed that the DNA of the cyclo(-pro-tyr) group was more intact than the DNA of the ethanol-induced group.

5.7 ANALYSES OF INFLAMMATORY MARKERS THROUGH qRT-PCR

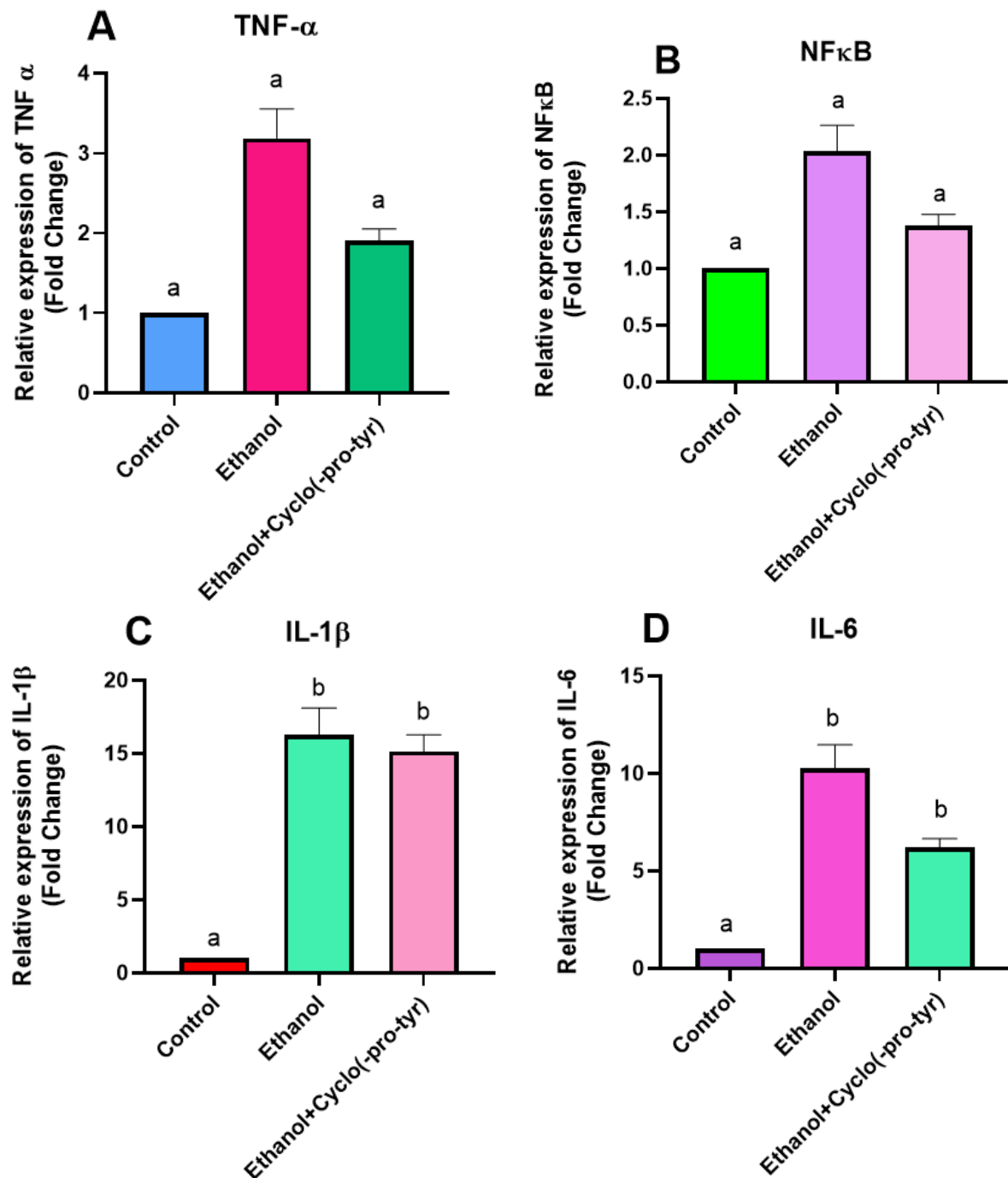


Fig. 5.11: Quantitative RT-PCR Analysis of Inflammation-Related Genes in the zebrafish of control and ethanol administrated Zebrafish for 3 days as acute liver injury study. The zebrafish were treated with or without 0.5% ethanol or Cyclo(-pro-tyr). Data are presented as the mean $\pm$ SEM ($n = 3$); 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).

When the ΔCT value exceeds 1, it indicates an upregulation in 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 following ethanol administration in zebrafish. This suggests that ethanol treatment may induce inflammation, as NFkB stimulates the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines, and also plays a role in regulating inflammation. IL-6 is a well-known immune-modulating cytokine with potential pro-inflammatory properties that can activate both the innate and adaptive immune systems. IL-1 β serves as a crucial mediator of the inflammatory response, while TNF α is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation (Baolin Xu *et al.*, 2020).

When the ΔCT value is less than that of ethanol treatment but still higher than the control, it suggests that the addition of cyclo(-pro-tyr) reduces the expression of inflammatory markers compared to ethanol treatment alone. This reduction implies that cyclo(-pro-tyr) may mitigate the inflammatory response induced by ethanol. Ethanol treatment alone appears to increase the expression of inflammatory markers, indicating an induction of inflammation. However, when cyclo(-pro-tyr) is 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-alpha (TNF α) is decreased compared to ethanol treatment alone. This suggests that cyclo(-pro-tyr) may possess anti-inflammatory effects, as it reduces the expression of NFkB, IL-6, IL-1 β , and TNF α genes induced by ethanol treatment alone.

5.8 DISCUSSION

Goessling *et al.*, (2015), Lin *et al.*, (2015), Park *et al.*, (2020), and other studies highlighted the adaptability and efficacy of zebrafish as an animal model for studying liver diseases, such as ethanol-induced liver injury ALD and NAFLD. Importantly, all that was required for a model to assess a drug's efficacy was for it to be exposed to either ethanol or cyclo(-pro-tyr) therapy for three days in zebrafish. This suggests that it might be applied to high throughput in vivo drug screening to

identify new drugs that could protect the liver from ethanol-induced acute hepatic damage (Benić *et al.*, 2022). Using this model system, they found that riboflavin, TES-1025, or NR supplementation completely prevented acute liver damage caused by ethanol exposure. The intriguing possibility that hepatocytes shielded from ethanol-induced acute hepatic injury could prevent the development of alcoholic steatohepatitis was also raised by their findings (Park *et al.*, 2020). Additionally, dietary supplementation of particular compounds during binge or heavy alcohol drinking might lessen the severity of hepatic injury.

Models of alcohol-induced acute liver disease are very useful for quickly identifying medications that may be beneficial for treating ALD linked to acute hepatic injury brought on by excessive alcohol intake. In this investigation, the experimental zebrafish's blood and liver tissue had higher SGOT and SGPT levels when exposed to 0.2% ethanol (Park *et al.*, 2019). Consequently, this suggests that ethanol reduction causes liver injury. Similarly, Park *et al.*, (2020) have documented that the injection of 0.5% ethanol mirrored the elevated levels of SGOT and SGPT in the liver and serum of experimental and control zebrafish. According to Passeri *et al.*, 4 dpf zebrafish larvae exposed to 2% ethanol for 32 hours show an increase in hepatic *cyp2e1*, *sod*, and *bip* as well as about 80mM intracellular ethanol, suggesting that ethanol is metabolized and causes oxidative stress (Passeri, M.J. *et al.*, 2008). The study's findings indicate that BP-4, PABA, and PBSA may substantially change antioxidants SOD, GST, GSH, and MDA in zebrafish liver after exposure for seven to fourteen days. These findings imply that exposure to these organic UV filters may cause oxidative damage to Zebrafish liver and an increase in ROS production (Huang, X. *et al.*, 2020). As the first line of defense against oxidative stress, SOD can catalyze the transformation of O_2 into H_2O and H_2O_2 . The interaction of electrophilic groups of xenobiotics with sulphydryl groups of GSH to improve its hydrophobicity can be catalyzed by GST, a phase II detoxification metabolic enzyme (Huang, X. *et al.*, 2020). Moreover, GST exhibits GPx activity, which prevents lipid peroxidation. In this investigation, after seven days of exposure to three contaminants, SOD activity dramatically decreased in all groups as compared to the control group. The results were caused by Zebrafish that produced more ROS after exposure than SOD could have removed (Tao, Y. *et al.*, 2013).

Similarly, in our study the treatment with cyclo(-pro-tyr) has shown alterations in the activity of antioxidants such as SOD, GST and GSH in zebrafish liver.

In patients with a history of alcoholism, administering alcohol increased the effects on proinflammatory cytokines, resulting in elevated levels of TNF α , IL-1 β , and IL-6. Acute alcohol consumption increased the synthesis of IL-10 in human monocytes and decreased TLR8 or TLR4-induced TNF- α protein and mRNA expressions in an in vitro experiment comparing acute and chronic alcohol usage. However, long-term alcohol intake raised TNF α mRNA and protein levels without altering monocytes' production of IL-10 (Janicova *et al.*, 2020). Our study's results demonstrate the intricate relationship between ethanol exposure and inflammation as well as the possible medicinal uses of cyclo(-pro-tyr) in reducing inflammatory reactions. It is well established that ethanol causes inflammation via several pathways, including NF κ B activation and the production of pro-inflammatory cytokines such as TNF α , IL-1 β , and IL-6. The advancement of the disease and tissue damage may be facilitated by these inflammatory reactions. To sum up, this project's results highlight cyclo(-pro-tyr)'s potential benefits in preventing ethanol-induced acute liver injury and steatosis. We have demonstrated the potential of cyclo(-pro-tyr) as a medicinal agent in reducing the harmful effects of ethanol on the liver through thorough trial and analysis. These findings provide a hope for the creation of innovative therapies for alcohol-induced liver illnesses in addition to furthering our knowledge of the biology of hepatic steatosis. Furthermore, to confirm cyclo(-pro-tyr)'s therapeutic potential in pre-clinical and clinical settings, as well as to completely understand the processes underlying its efficacy, more investigation and clinical studies are necessary.

CHAPTER 6

SUMMARY AND CONCLUSION

This study investigated the possible protective impact of cyclo(-pro-tyr) against zebrafish liver damage caused by ethanol over an extended period of three days. The results convincingly demonstrated that cyclo(-pro-tyr) administration offers significant advantages in countering these harmful effects. The protein levels were regulated by cyclo(-pro-tyr), potentially preventing the liver's repair mechanism from becoming overloaded due to ethanol exposure. The group treated with cyclo(-pro-tyr) showed reduced liver enzyme activity (SGOT/SGPT) compared to the ethanol group, suggesting better liver function and less damage. The antioxidant enzymes SOD, CAT, GPx, GST, and GSH were all markedly increased in activity by cyclo(-pro-tyr). By doing this, the zebrafish exposed to ethanol are effectively protected from oxidative stress and its cells. Cyclo(-pro-tyr) demonstrated anti-inflammatory effects by reducing the inflammatory gene expression, including NF- κ B, IL-6, IL-1 β and TNF- α . This indicated its potential to alleviate the inflammatory response triggered due to ethanol exposure. Additionally, the histopathological examination also revealed that cyclo(-pro-tyr) reduced steatosis compared to the ethanol group. In conclusion, this study provides compelling evidence that the administration of cyclo(-pro-tyr) presents a promising therapeutic approach to protect against ethanol-induced acute liver disease. It reduces protein synthesis, enhances liver function, improves antioxidant defense, alleviates inflammation and prevents liver disease. However, further research is required to confirm these findings in clinical and pre-clinical settings and assess the safety and effectiveness of cyclo(-pro-tyr) for human use in managing alcoholic liver disease.

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