NATURAL REMEDIES FOR HEPATIC DISEASES

THESIS SUBMITTED

BY

AVISHEK MANDAL

DOCTOR OF PHILOSOPHY (PHARMACY)

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FACULTY COUNCIL OF ENGINEERING & TECHNOLOGY

JADAVPUR UNIVERSITY

KOLKATA-700032

INDIA

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Dedicated

To

My Beloved

Ma (Rina Rani Mandal)

&

Baba (Sushanta Kumar Mandal)

JADAVPUR UNIVERSITY

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2. Name, Designation & Institution of the Supervisor:

Prof. Sanmoy Karmakar

Professor of Pharmacology and Toxicology,

Dept. of Pharmaceutical Technology,

Jadavpur University,

Kolkata 700032

India

3. List of Publications:

- **Avishek Mandal**[1], Rudranil Bhowmik[1], Md. Adil Shaharyar[1], Kumar Anand[1], Pallab Mandal[1], Jamal Akhtar[2], Samit Karmakar[3], Sanmoy Karmakar[1]*. "***The Protective Action of Earthworm Protein Powder/ Mwco 50 Kda Fraction On Experimental Hepatic Damage: An Invivo/Invitro Approach Involving Inflammatory Tnf-A And Il-6***" **European Chemical Bulletin (ISSN: 2063-5346),2023; Volume -12, Special Issue-5(Part-A): Page: 937 – 953**

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Book Chapter:

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- **Avishek Mandal**1, Sanmoy Karmakar1*. ***"Ameliorative Effect of The Combination of Natural Formulation Goat's Milk and Indian Earthworm Extract Powder On Ethanol-Induced Hepatotoxicity"*** in the International Conference on "Latest Trends on Applied Science, Management, Humanities and Information Technology" held at Sai College, Bhilai, Chhattisgarh, India on 26-27 May 2023.

PROFORMA – 1

"Statement of Originality"

I, Sri Avishek Mandal registered on 3rd November 2016 do hereby declare that this thesis entitled "Natural Remedies for Hepatic Diseases" contains literature survey and original research work done by the undersigned candidate as part of Doctoral studies.

All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have fully cited and referred all materials and results that are not original to this work.

I also declare that I have checked this thesis as per the "Policy on Anti Plagiarism, Jadavpur University, 2019", and the level of similarity as checked by iThenticate software is 9 %.

Avishek Mandal

Signature of the Candidate

Date: 10/01/2024

Sanmoy Karmakar 10/01/2024

Certified by Supervisor

(Signature with date, seal)

DR. SANMOY KARMAKAR
PROFESSOR (Pharmacology & Toxicology)
Dept. of Pharmaceutical Technology
JADAVPUR UNIVERSITY, Kolkata-32

PROFORMA - 2

CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled ***"Natural Remedies for Hepatic Diseases"*** *submitted by* ***Sri. Avishek Mandal****, who got his name registered on 3rd November, 2016 [MISC. 33140 (Ref. no. D-7/E/817/16)] for the award of Doctor of Philosophy (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervision of* ***Prof. Sanmoy Karmakar*** *and that neither his thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.*

Sanmoy Karmakar
10/01/24

DR. SANMOY KARMAKAR
PROFESSOR (Pharmacology & Toxicology)
Dept. of Pharmaceutical Technology
JADAVPUR UNIVERSITY, Kolkata-32

Prof. Sanmoy Karmakar (Supervisor)
Professor of Pharmacology and Toxicology,
Dept. of Pharmaceutical Technology,
Jadavpur University,
Kolkata 700032.
India

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Kolkata, January, 2024 Avishek Mandal

PREFACE

This research work is carried out in partial fulfillment of Doctor of Philosophy (Pharmacy). The current research work entitled "Natural Remedies for Hepatic Diseases" was used as a hepatoprotective natural product.

Liver disease is one of the most common lifestyle diseases these days, affecting one in every three people. It is one of the greatest health challenges of the 21st century. Although this condition is common, easily recognised, yet no proper comprehensive treatment unveiled till now. It is usually asymptomatic and often leads to serious complications such as fatty liver, jaundice, organ failure, fibrosis, acute liver failure, chronic liver damage, and metabolic imbalance syndrome if left unattended. In addition, excessive use of medications eg NSAIDs, exposure to industrial chemicals, and alcohol abuse can cause complete damage of liver. Here earthworm crude extract and 50 kDa MWCO of earthworm extract proteins were isolated. Then different concentrations were made especially at 25, 50, 100, and 200 mg/ml, and used in the in-vivo model. Moreover, it was found to significantly increase its expression in primary hepatocytes with 25, 50, 75, 100 ug/ml in an in vitro model. Crude protein is first extracted from *Metaphyre posthuma* in protein extraction. Additionally, for 50 MWCO we used Sephadex G 75, Viva spin column, 50-kDa nominal molecular weight cut-off (MWCO), 3-kDa MWCO column, Centriprep® Centrifugal Filter 10 kDa, and for fast protein purification after dialysis. FPLC is performed. For the protein test, total protein assay, bradford assay, and lowry protein assay are done. In addition, crude protein, protein below 50 MWCO, and 6.023 kDa were analyzed by SDS PAGE then trypsin digestion and MALDI TOF MS was performed, and some known and also some unknown sequence of the peptide were found. It could be hypothesized that this protein or peptide may have hepatoprotective activity.

High doses of paracetamol, carbon tetrachloride, and ethanol were applied in an in-vivo model to evaluate the hepatoprotective activity of *Metaphire posthuma* protein powder (MP). Acute and chronic model of toxicity, especially the liver fibrosis models were used in this study. Standard medicine especially silymarin, ursodeoxycholic acid, and goat milk as vehicle were used accompanying with earthworm protein.

During the study, wister albino rats were divided into four groups: control, paracetamol-treated, paracetamol alone with EP(earthworm extract protein powder), and paracetamol

along with silymarin. The animals showed significant elevations in alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, LPO or MDA, total bilirubin in plasma, mRNA expressions of TNF-α and IL-6 in liver tissue while the levels of CAT, SOD, and GSH in plasma was found decreased in the paracetamol group. The separated MWCO 50 kDa protein from EP showed significant reduction of AST and ALT, ALP, and bilirubin in primary hepatocyte culture and increased percentage viability of cells. The histological examination showed disruption of liver histoarchitecture in the paracetamol group, but restoration was found in the paracetamol-EP group. The in-vivo study, restoration of biochemical parameters, oxidative stress markers modulation, changes in mRNA expressions, and histopathological findings in the paracetamol-EP and paracetamol-silymarin groups. The acute liver damage due to paracetamol is attributed to oxidative stress in the animal model. EP decreased oxidative stress and inflammation, slowing down the liver damage progression caused by paracetamol. EP activities involving cytokine expressions using lower molecular weight protein fractions are of scientific importance with correlation to real-time use.

Another study aimed to evaluate the effectiveness of pre-treating primary hepatocyte cells with earthworm extract to prevent damage from carbon tetrachloride (CCl_4). Cell viability, AST, ALT, MDA levels, TNF-α activities, and hepatoprotective activity of Earthworm extract protein powder (EEP), goat milk powder (GP), and Silymarin (SLM) were assessed in-vitro on rat primary hepatocyte culture. The study found that CCl4-induced cytotoxicity is influenced by oxidative stress. The hepatoprotective activity of EEP and EEP+GP was identified, and lipid peroxidation assays showed that MDA levels were inhibited by EEP and GP treatment. The study concluded that EEP and GP function as efficient hepatoprotective agents, preventing CCl4 treatment-induced primary hepatocyte toxicity.

Ethanol consumption is responsible for 60-80% of liver disease deaths, including Alcoholic liver disease (ALD). Et-OH (ethanol) causes oxidative stress and inflammation, leading to immunological dysfunction and cytokine imbalance. Addressing Et-OH-induced liver damage can be achieved using GSH and CAT enhancers. ALD disrupts bile acid metabolism and increases chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA). Goat milk (GM) is a suitable delivery medium for MP, which can produce synergistic effects against hepatic damage. Acute toxicity studies show a maximum tolerated dose of MP (Metaphire posthuma protein powder) is over 2 g/kg in rats, while sub-acute toxicity evaluation shows no toxic effects. MP+GM reduces pro-inflammatory markers and protects against Et-OH-induced

hepatotoxicity by increasing CAT, GSH, Albumin, total bilirubin, cholesterol, and suppressing triglyceride activity.

Chronic liver disease, a major cause of severe hepatotoxicity, is primarily mediated by liver fibrosis. In our study the potential of Earthworm Extract Protein (EEP) from *Metaphire posthuman* protein *(*MP*)*, was investigated against CCl_4-induced liver fibrosis. The rats were exposed to CCl_4 for 12 weeks, and MP was administered twice weekly. The effects of MP were evaluated by examining parameters associated with liver fibrosis, such as body weight, histological changes, biochemical indicators, and antioxidant activity. The study found that MP modulate the expression of AKT, BAX, α-SMA, Nrf-2, IL-6, STAT-3, TNF-α, and IL-4 pathways, inhibited liver function testing, activated antioxidants, and prevented an increase in cholic acid in blood plasma. The findings suggest that MP could be a candidate for natural remedy of chronic liver damage.

Department of Pharmaceutical Technology
Jadavpur University
Kolkata-700032

Avishek Mandal
.....
(AVISHEK MANDAL)

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List of Abbreviations

Short Form	Full Name
µg	Microgram
ACR	Alkaline Copper Sulphate
AD	Anno Domini
ALT	Alanine transaminase
ANOVA	Analysis of Variance
AP	Ammonium Persulphate
APTT	Activated partial thromboplastin time
AR	Analytical Reagent
AST	Aspartate transaminase
B.C.	Before Christ
BApNA	N-Benzoyl-DL-arginine-4-nitroanilide hydrochloride
Bcl2	B-cell lymphoma 2
BCPV	Before carrageenan paw volume
BSA	Bovine serum albumin
CaCl2	Calcium chloride
CAT	Catalase
CBB G-250	Coommasie brilliant blue Green-250
CBC	Complete blood count
CCF-1	Coelomic cytolytic factor-1
c-DNA	Complementery- Deoxy Ribo Nucleic Acid
CF	Coelomic fluid
CFU	Colony forming unit
CMV	Cucumber mosaic virus
$CuSO_4$	Copper sulphate
DIC	Disseminated intravascular coagulation
DMSO	Dimethyl sulphoxide
DNA	Deoxyribo Nucleic Acid
Dnase	Deoxyribonuclease
DPPH	2,2-Diphenyl 1-picryl hydrazyl
DPX	Dibutyl Phathalate Xylene

Short Form	Full Name
DRDE	Defence Research and Development Establishment,
ds DNA	Double stranded Deoxyribo Nucleic Acid
DTNB	5,5- Dithio bis 2- nitro benzoic acid
EC50	Effective concentration 50
ECFP	Earthworm coelomic fluid protein
ECLT	Euglobulin clot lysis time
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EE	Earthworm extract
EFE	Earthworm fibrinolytic enzyme
EfP	*Eisenia fetida* protease
EF-SP1	*Eisenia foetida* Serine Protease 1
EF-SP2	*Eisenia foetida* Serine Protease 2
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbant assay
e-PA	Earthworm-plasminogen activator
ERK1/2	Extracellular signal-regulated kinase 1/2
NAFLD	Non Alcoholic Fatty Liver Disease
NASH	Non-alcoholic Steatohepatitis
HCC	hepatocellular carcinoma
CLDS	chronic liver diseases
DAAS	direct-acting antivirals
Da	Dalton
ROS	reactive oxygen species
Bcl-2	B-cell lymphoma 2
BAX	Bcl-2-associated X protein
IL6	interleukin-6 (IL-6)
TNF-α	tumor necrosis factor-alpha (TNF-alpha)
mRNA	Messenger RNA
kDa	kilodalton
TGFβ	Transforming growth factor-beta

Short Form	Full Name
AMPK	AMP-activated protein kinase
ATP	Adenosine tri-phosphate
ADP	Adenosine di-phosphate
PH	Partial hepatectomy
HSCs	Hepatic stellate cells
ALP	alkaline phosphatase
ALT	alanine transaminase
AST	aspartate aminotransferase
GGT	gamma-glutamyl tansferase
SOD	superoxide dismutase
↓	decrease
↑	increase
BMI	body mass index
TG	triglyceride
TC	total cholesterol
LDL-C	low-density lipoprotein cholesterol
LP	lipoprotein
PPARγ	peroxisome proliferator-activated receptor γ
MDA	malondialdehyde
TBARS	thiobarbituric acid reactive substances
Inos	inducible nitric oxide synthase
COX-2	cyclooxygenase-2
NRF2	Nuclear factor erythroid 2-related factor 2
GM	Goat Milk
GP	Goat Milk PROTEIN POWDER
Et-OH	ETHANOL
ALD	Alcoholic liver disease (ALD)
EE	EARTHWORM EXTRACT
CDCA	chenodeoxycholic acid (CDCA)
DCA	deoxycholic acid (DCA
CYP2E1	Cytochrome

Short Form	Full Name
CAT	Catalase
GSH	Glutathione
LFT	liver function tests
NASH	Non-alcoholic Steatohepatitis
HCC	hepatocellular carcinoma
ALD	alcoholic liver disease
Da	Dalton
RT-PCR	Real time polymer chain reaction
MAPK	Mitogen-activated protein kinase
TCM	Traditional Chinese medicine
CF	coelomic fluid
PP	Purified peptides
IP	Intraperitoneal injection
PO	Per OS (orally)
PCM	Paracetamol
EP	Earthworm protein powder
MWCO	Moloecular weight cut off
LPO	Lipid per oxidation
ALP	Alkaline phophate
ROS	Reactive oxygen species
MCP-1	Monocyte Chemoattractant Protein-1
MIP-1α	Macrophage inflammatory protein
VEGF	Vascular endothelial growth factor
SILY	Silymarin
MDA	Malondialdehyde
Dntp	Deoxynucleotide triphosphate
Dnase	Deoxyribonuclease
DTT	Dithiothreitol
cDNA	Complementary DNA
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
mM	Milli molar

Short Form	Full Name
Um	Micro molar
ul	Micro-litter
(NH4)2SO4	Ammonium sulfate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
CO2	Carbondioxide
HCl	Hydrochloric acid
DMSO	Dimethyl sulfoxide
H & E	hematoxylin and eosin
KP	Kupffer cells
NAPQI	N-acetyl-p-benzoquinone imine
mt-DNA	Mitochondrial DNA
EEP	Earthworm extract protein powder
UDCA	Ursodeoxycholic acid
ALI	Acute liver injury
DILI	Drug induced liver injury
LD50	Lethal dose 50
DMEM	Dulbecco Modified Eagle Medium
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
GP	Lyophilized goat milk powder
SLM	Silymarin
HBSS	Hank's Balanced Salt Solution
EDTA	Ethylenediaminetetraacetic Acid
NaCl	Sodium chloride
KCl	Potassium chloride
FBS	Fetal bovine serum
TCA	Tri chloro acetic acid
GM	Goat milk
EtOH	Ethanol
ELISA	Enzyme-linked immunosorbent assay
TMB	3,3′,5,5′-Tetramethylbenzidine

Short Form	Full Name
LC-ESI-MS	Liquid Chromatography-Electrospray Ionization-Mass Spectrometry
QC	Quality control
LLOQ	Lower limit of quantitation
LQC	Lower quality control
MQC	Medium quality control
HQC	High Quality control
hr	Hour
FPLC	Fast protein liquid chromatography
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
SD	Standard Deviation
MP	Metaphire posthuma protein powder
GM	Goat milk
Ethanol	Et-OH
ALD	Alcoholic liver disease
CYP 2E1	Cytochrome P450 2E1
TNF-α	Tumour Necrosis Factor alpha
IL-6	Interleukin-6
GSH	Glutathione
CAT	Catalase
CDCA	Chenodeoxycholic acid
DCA	Deoxycholic acid
EE	Earthworm extracts
AST	Aspartate aminotransferase
ALT	Alanine transaminase
LCA	Lithocholic acid
TCM	Traditional Chinese Medicine
OECD	Organisation for Economic Co-operation and Development
HPLC	High-performance liquid chromatography
IS	Internal standard
PBS	Phosphate buffer

Short Form	Full Name
NMWCO	Nominal molecular weight cut-off.
kDa	Kilodalton
CPCSEA	Committee for the Monitoring and Control of Experiments on Animals
IAEC	Institutional Animal Ethics Committee
CNS	central nervous system
mg	milligram
kg	kilogram
µm	micrometre
g	gram
NIH	National institute of health
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Mm	millimolar
rpm	rotational per minute
ICH	The International Council for Harmonisation of Technical Requirements of Pharmaceuticals for Human Use
LOD	Limit Of Ditection
LOQ	Limit Of Quantification
ANOVA	One-Way Analysis Of Variance
SD	Standard Deviation
%	Percentage
U	Unit
dl	Decelitter
L	Litter
µl	Microlitter
ARRIVE	Animal Research Reporting of In Vivo Experiments
TG	Triglycerides
GSSG	Glutathione disulfide

CHAPTER I

INTRODUCTION

1. Introduction

The liver is considered as one of the essential organs in the human body since it performs a crucial function in several physiological processes. The liver is in the superior right quadrant of the abdominal cavity, immediately inferior to the diaphragm. The organ in context is among the biggest in size inside the human body. The liver performs a multitude of vital tasks [1]. The digestive system is responsible for the breakdown and absorption of nutrients derived from the ingested food, including macronutrients such as carbohydrates, proteins, and fats. The liver is crucial in eliminating toxins and waste substances from the bloodstream. The liver serves as a repository for essential compounds, including glycogen, vitamins, and minerals [2]. The liver is responsible for the synthesis and secretion of bile, which plays a crucial role in facilitating the breakdown and assimilation of dietary lipids inside the small intestine. This organ synthesises various proteins, such as blood clotting factors and albumin. The liver plays a crucial role in regulating blood glucose levels via its capacity to store and release glucose in response to physiological demands. Liver also participates in immune system, being involved in the process of eliminating bacteria and other exogenous particles from the bloodstream [3]. However, such a vital organ is susceptible to various disorders, such as hepatitis (hepatic inflammation), cirrhosis (hepatic tissue scarring), fatty liver disease, liver cancer, and other related ailments. These disorders may arise due to several circumstances, including viral infections, excessive alcohol intake, obesity, and genetic predisposition. The liver is an organ characterised by its unique capacity for regeneration[4]. The regenerative capacity of the liver enables the feasibility of partial liver transplants from living donors since it has the ability to repair and replace damaged tissue [5]. To evaluate the functionality and condition of the liver, medical practitioners may request for blood examinations, specifically liver function tests (LFTs), which measure the concentrations of enzymes, proteins, and bilirubin present in the bloodstream. Imaging modalities such as

ultrasounds, computed tomography (CT) scans, or magnetic resonance imaging (MRI) might also offer insights on the structural characteristics of the liver. In instances of severe hepatic disorder or insufficiency, the transplantation of a liver may become imperative. During the surgical procedure known as a liver transplant, the process of replacing a diseased or injured liver with a viable liver obtained from either a dead or living donor takes place. Adhering to a health-conscious lifestyle may contribute to the prevention of liver disease. The recommended preventive measures include the restriction of alcohol intake, the avoidance of high-risk behaviours that may result in viral hepatitis, adhering to a well-balanced dietary regimen, and engaging in regular physical activity. Viral hepatitis, including hepatitis B and C, can potentially cause chronic liver disease and represents a substantial worldwide health issue [6]. The administration of vaccinations can prevent hepatitis B, whereas hepatitis C may be effectively managed with the use of antiviral medications. The liver is an essential organ that plays a critical role in sustaining general health, and it is of utmost importance to ensure the proper functioning of this organ [7].

1.2 Liver Disease in Global Situation and Status in India

Two million people die every year from liver disease, which is 4% of all deaths (1 out of every 25 deaths in the world). About two-thirds of all liver-related deaths happen to males. Cirrhosis and liver cancer are the main causes of death. Acute hepatitis is responsible for a smaller number of death [8]. Around the world, viral hepatitis, alcohol, and non-alcoholic fatty liver disease are the main causes of cirrhosis. Acute hepatitis, primarily caused by hepatotropic viruses, is becoming a significant global health concern due to drug-related liver damage. Hepatitis B and C are major contributors to liver disease worldwide. Viral hepatitis and alcoholic liver disease are major causes of liver damage, with vaccinations, antiviral treatments, and public health campaigns being the available remedies. Non Alcoholic Fatty Liver Disease (NAFLD) is a growing concern globally. It's associated with obesity, sedentary

lifestyles, and poor dietary habits. In some cases, it can progress to Non-alcoholic Steatohepatitis (NASH), which is more severe and can lead to cirrhosis and liver cancer [9]. Cirrhosis is a common endpoint for various liver diseases, including viral hepatitis, alcoholic liver disease, and NASH. It is characterized by the scarring of liver tissue and impaired liver function. Liver cancer, especially hepatocellular carcinoma (HCC), is a major global health issue. Chronic liver diseases, such as viral hepatitis and cirrhosis, are significant risk factors for developing liver cancer [11]. Access to healthcare, including early diagnosis and treatment, varies worldwide. In some regions, limited resources and infrastructure can hinder the management of liver diseases. Various international organizations, such as the World Health Organization (WHO), are actively working to combat liver disease through vaccination, awareness campaigns, and improved healthcare access [12]. Liver transplantation is a treatment option for end-stage liver disease. However, donor organs' availability and transplantation's affordability and chronic use of immunosuppressant can be challenging issues in many countries [12]. Preventing liver disease involves lifestyle changes like healthy eating, exercise, and alcohol moderation. Research explores new treatments and therapies for viral hepatitis and liver cancer management. Liver diseases are becoming a significant public health priority in India, contributing to 18.3% of global liver disease-related deaths in 2015. The burden of cirrhosis and its complications, collectively known as chronic liver diseases (CLDs), has increased since 1980, compared to China, where it remains stationary and shows downward trends [13]. India faces limited quality of available epidemiological data resources on liver disease. Still, evidence suggests the increasing impact of liver diseases on the country's economy, healthcare resources, and premature death and disability. The cultural-lifestyle transition in India, with the adoption of a Western diet and sedentary habits, has led to a spectrum of liver diseases that shows signs of a rapid switch,

including increasing the importance of alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) as causes of liver disease.

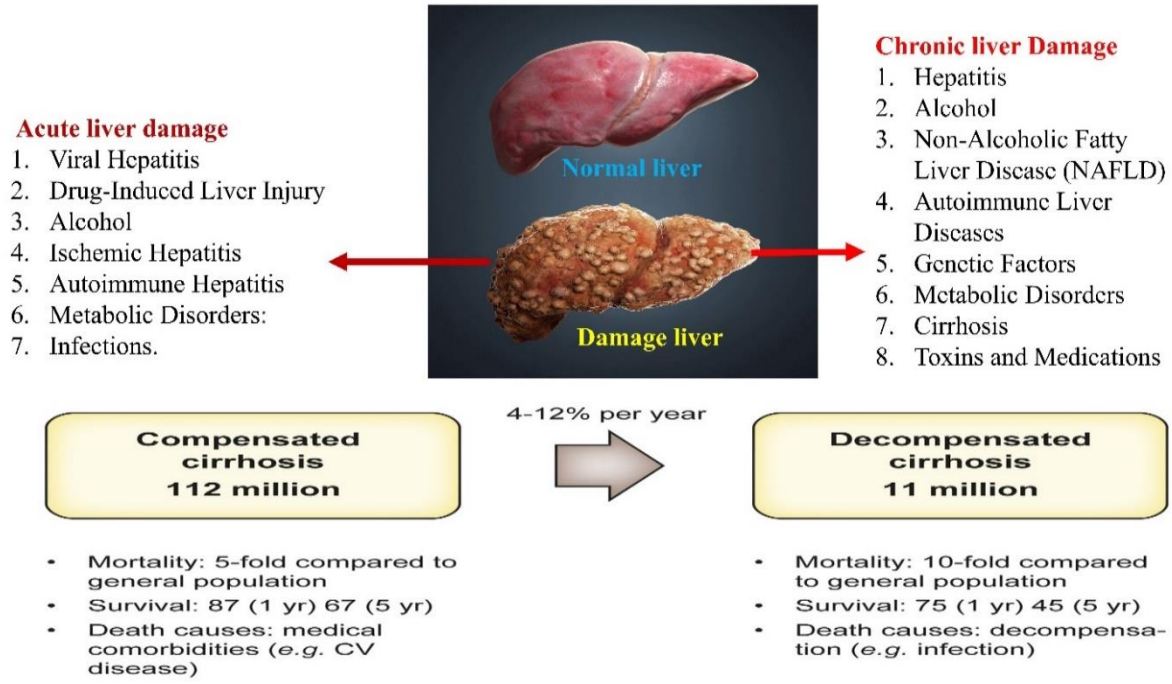

Figure 1. Acute and chronic liver damage

1.3 Functions of the liver

The liver is a complex organ with two primary lobes, the right and left, and two smaller lobes, the caudate and quadrate lobes. It is divided into functional segments with blood supply and bile drainage [1]. The liver tissue is organized into lobules, which are hexagonal and consist of hepatocytes, the liver's functional cells. Hepatocytes perform various metabolic functions, processing and regulating carbohydrates, fats, proteins, and substances in the bloodstream. They also produce bile. Sinusoids, tiny blood vessels between hepatocytes within the lobules, allow for the exchange of nutrients, oxygen, and waste products between the hepatocytes and the blood [14]. Kupffer cells, a type of macrophage, help remove foreign substances from the bloodstream . Hepatocyte-produced bile travels through bile ducts to the gallbladder or directly to the small intestine. The liver receives oxygenated blood through the hepatic artery, providing oxygen and nutrients to the liver cells [15]. Nutrient-rich but

oxygen-depleted blood from the digestive organs is delivered to the liver via the portal vein. The liver has a lymphatic system that removes extra fluid and waste from the liver tissue and connective tissue to support it. It is a metabolic powerhouse, regulating blood sugar levels, preventing pollution, and removing toxins from the body [16].

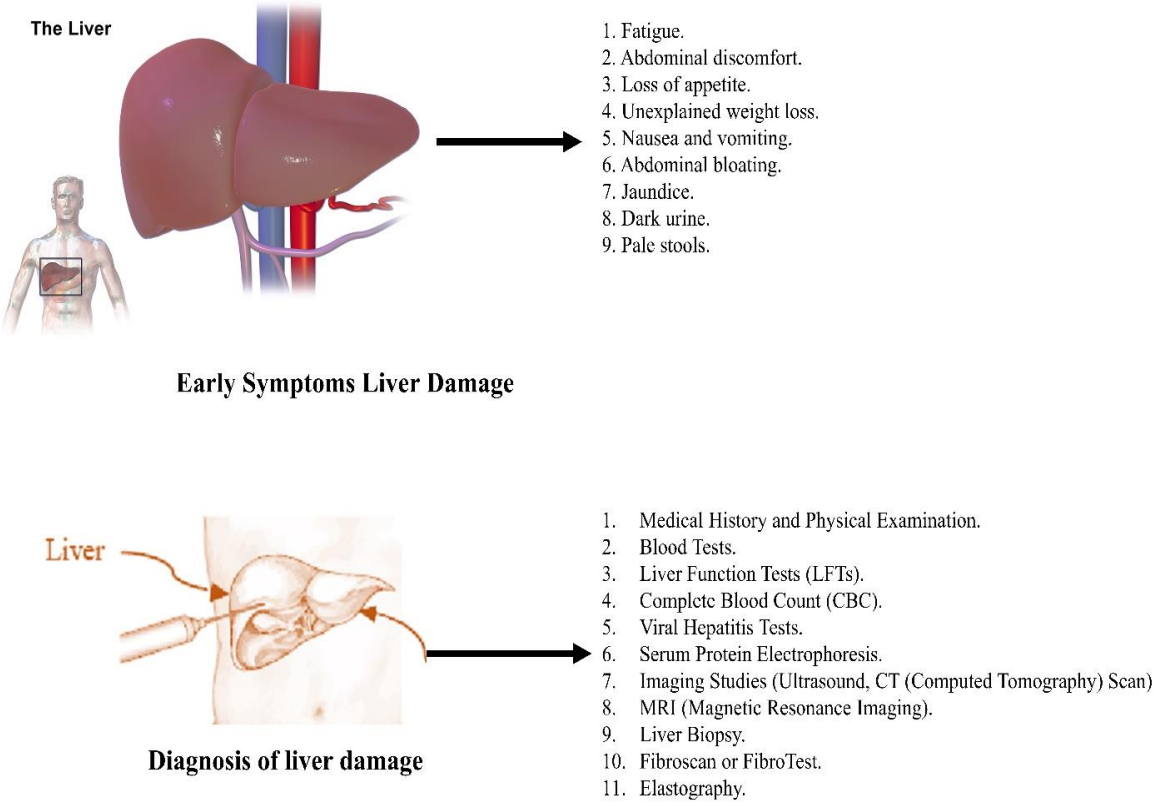

Figure 2. Early liver damage symptoms and diagnosis:

1.4 Reasons for Liver damage

Different types of liver diseases affect people at different times. However, it can be seen that we often do not realise that our liver is being damaged because liver disease is often asymptomatic. Among them, jaundice, fatty liver, fast food, alcohol, side effects of various drugs, and taking more painkiller medications affect the liver the most. If liver disease is not cured of its own, as such no effective medicine has been developed to cure liver disease. Various Ayurvedic medicines from plants have been found to influence the liver positively [17]. However, patients often do not use them due to lack of proper evidence. Presently, many

types of proteins, peptides, and enzymes have been scientifically found to have protective effect on the liver [18]. However, extracting and purifying these proteins is not at all use friendly. It will be interesting to mention that traditional healers have been using typical type of insects and earthworms as a remedy for liver diseases [19]. Many of these traditional treatments are been used from time immemorial. Considering the locally believed evidence based behind these treatments, reverse pharmacological investigation might help to understand the reasons for popularity of these traditional believe system.

1.5 Bile acid imbalance

Bile acids, produced by the liver and stored in the gallbladder, are essential for the digestion and absorption of dietary fats. In the event of imbalanced in these functions, they can lead to liver damage, cholestasis, hepatocyte injury, liver fibrosis, and cirrhosis. Cholestasis is a condition where bile flow from the liver is impaired, causing damage to hepatocytes [20]. Hepatocyte injury can result from excessive bile acids, leading to inflammation and liver damage. Liver fibrosis can progress to cirrhosis, a serious condition where the liver loses its normal function [21]. Bile acids also play a role in detoxification, but liver damage can impair its ability to perform essential functions. Systemic effects of liver damage include symptoms like jaundice, fatigue, itching, and alterations in metabolic and hormonal processes [20,21]. Diagnosis and treatment of bile acid imbalances are crucial to prevent or mitigate damage.

1.6 Proteins for the Liver Ailment

Several new medications and treatment approaches were being developed and tested for various liver conditions, including hepatitis, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and liver cancer. New antiviral medications have been developed to treat hepatitis B and C. These drugs, such as direct-acting antivirals (DAAs) for

hepatitis C, have shown high cure rates and fewer side effects than older treatments. Research into medications for NASH and NAFLD is ongoing [22]. Protein research focuses on identifying specific proteins or peptides that can protect the liver from damage or support its function. Some research areas include cysteine-rich peptides, milk-derived peptides, egg white proteins, plant-derived proteins, fish-derived proteins, fish collagen, lactic acid bacteria, and insect-derived proteins. Cysteine is an amino acid precursor to glutathione, a potent antioxidant in the liver [23][24]. Milk-derived peptides, such as casein and whey, have been studied for their potential to improve liver function and protect against liver damage. Egg white proteins, such as ovotransferrin, have been investigated for their antioxidant and anti-inflammatory properties [26]. Plant-derived proteins and peptides include soy protein, rice bran protein, wheat germ protein, has also been considered for reseach [27]. Fish collagen peptides have been explored for their hepatoprotective effects, including their ability to reduce liver fibrosis and inflammation [28][29]. Fish-derived oligopeptides have also been studied for their hepatoprotective properties [27][28][30]. Despite being promising, many such findings are still experimental, and further clinical research is needed to confirm their potential benefits for liver health. Innovations in drug delivery methods, such as nanoparticles and targeted drug delivery, are being explored to enhance the effectiveness and reduce side effects of liver disease treatments.

CHAPTER -II

REVIEW

OF

LITERATURE

2.1 Hepatoprotective activity of some reported biological proteins and their sources

Investigations suggest that a few biological protein sources can cure liver protection and anti-fibrotic activities, with marine compounds like *Psolus phantapus, Celleporina surcularis,* and *Actinostola callosa* showing anti-inflammatory properties and potent H1 receptor inhibitory function [31]. Chittapon Jantararussamee *et al*. reported that probiotics (such as *Lactobacillus paracasei, Lactobacillus casei,* and *Weissella confuse*) have improved chances in recent days to prevent and cure different forms of disorders and protect the liver [32]. In early studies, casein glycomacropeptide, which was bound to amino acids, had antioxidant properties that protected the liver [32]. Heavy metals like lead, mercury, and cadmium disrupt liver cells. *Trichurus spiralis,* a marine-derived fungus has been reported to protect the liver from heavy metal poisoning [33]. Antibacterial and antioxidant potential in milk peptides assist in fighting hepatic disorders, according to the research [33][34]. Buffalo milk (globally second-most milk type produced) includes antioxidant and antimicrobial peptides. Abundance of free radicals, which are often generated during biological oxidation, may harm biological systems. Whey protein can improve hepatitis and portal fibrosis [35]. A model on laboratory animals showed that high dose of ethanol cause oxidative damage in microsome and mitochondria. Ethanol (3gm/kg body weight) produced hepatotoxicity in rats after 15 days; however, fish sardinelle (Sardinella aurita) protein from the heads and viscera preserves hepatic and renal function [36][37]. Many metabolic activities in the liver allow toxic agents to directly injure liver cells, including hepatocytes [38]. Three different proteins (molecular weight: 1860.85 Da, 1477.63 Da, and 903.04 Da) in camel milk have tested cytotoxicity against the hepG2 cell line and showed positive activity in RT-PCR analysis of superoxide dismutase and catalase genes [39]. Chang-Feng Chi *et al*. hydrolyzed three proteins/peptides from bluefin leatherjacket (*Navodon septentrionalis*) fish and found strong antioxidant and peroxidation inhibition [40]. Peptides from blue mussels (Mytilus edulis) are antioxidant in nature [40].

The hepatotoxic chemical usually activates kupffer cells, attracts macrophages and produce ROS, causing lipid peroxidation and membrane breakdown [43]. Insect proteins with hepatoprotective nature modulated the Bax/Bcl-2 ratio and protected liver cells through the MAPK/caspase-3 pathway [44]. Metallothionein protects cells from heavy metals and oxidative stress [45][46]. Recently, protein or peptide treatments for hepatic disorders have shown great liver cell regeneration. According to Selvakumari Ulagesan *et al.* seven proteins found in freshwater land snails (*Cryptozona bistrialis*) that suppress harmful bacterial and fungal cultures [47]. Golden grey mullet (*Liza aurata*) protein comprises glutamic acid, glutamine, lysine, proline, leucine, and glycine. A protein fraction of *Liza aurata* (3–20 kDa) showed hepatoprotective efficacy in both *in vitro* and *in vivo* against paracetamol in 45-day animal models [48]. Nowadays, protein-derived peptides are fascinating due to their numerous pharmacological and defensive mechanisms [49]. Goat's milk, snail (*Bellamia bengalensis*) extract, and cod skin collagen peptides are hepatoprotective, antimicrobial, cytotoxic, anti-tumour, anti-inflammatory, antileukemic, antineoplastic, and antiviral [50][51]. Goat milk is rich in functional and health-promoting ingredients like proteins, oligosaccharides, fatty acids, and phospholipids. These substances prevent chronic diseases, particularly cardiovascular diseases, and promote gastrointestinal health. Calcium, a key cellular signaling component, has been shown to help reduce obesity. To enhance the health benefits of goat milk, it's suggested to increase its bioactive components and minerals, thereby improving its functional attributes [52][53][54]. Krill (*Euphausia superba*) protein 1–3 kDa fraction protects rodent livers against ethanol-induced liver injury and downregulates TNF-alpha and IL-6 mRNA expression [55]. The Korean government has registered insects as medication in the Ministry of Food and Drug Safety to treat inflammatory disorder, hepatic cancer, liver cirrhosis, and hepatitis [56]. In animal models of non-alcoholic fatty liver disease (NAFLD), insect (*Forsythia viridissima*) protein extracts show promise [57]. Another

study discovered that the protease enzyme lowers liver damage caused by TGF-beta/Smad and raises the expression of antioxidant proteins [58].

The earthworm-based anti-thrombus technique is difficult and generates low-purity protein. *Lumbricus rubellus* earthworm and *Perinereis linea* lugworm had fibrinolytic activity already reported in several scientific journals [59][60]. Further, another investigation showed lugworm hydrolysate-containing proteins with varying molecular weights affected damaged hair's permanent wave treatment. [61]. In general, AMPK activation switches off ATP-consuming processes and ATP-generating pathways. Partial hepatectomy (PH) increases AMPK phosphorylation and hepatocyte growth. Through any G1/S transition phase action, AMPK deletion decreases liver development [64]. Other effects of AMPK activation include hepatocyte polarization [65]. Oral supplementation with marine fish and fish oil may activate AMPK and alter hepatocyte polarisation indicators, increasing liver function recovery following hepatectomy [66]. In Asian traditional medicine, Cordycepin, a *Cordyceps militaris* (entomogenous fungus) metabolite, is a biologically active antioxidant and anti-inflammatory decreases Smad phosphorylation and phosphate to inhibit TGF-1 signaling and HSC activation [67][68]. Cordyceps polysaccharide reduces acute liver failure by regulating pro- and anti-inflammatory factors and reducing apoptosis [69]. Natural proteins and powerful nutrients are found in insects. Several studies involving insect protein reported that about 40% to 75% is dry weight and amino acids are 76% to 96 % digestible [31][32][38][49][62][69]. Marine microorganisms, particularly fungi, exist in the marine environment and produce unique secondary metabolites, making them a potential source of pharmacologically active metabolites [70]. Marine fungi and earthworms have potential therapeutic properties, including anti-tumour activity in cell lines, and have been used as toxicological models for heavy metals with limited research [32][69][70][71].

The present study explores the impact of dietary intake on overall well-being, focusing on earthworm extract protein/ enzymes and animal milk, this is important since despite centuries of use, the advantages of these organisms remain ambiguous.

Table 1. Available Traditional Therapies for Liver Disease from Animal Source

Hepatoprotective source	Protective Activity	References
Corbicula fluminea Muller	Bioactive Agent hepatoprotective	[31]
A. cerana honey	AST↓ ALT ↓ GGT↓ bilirubin ↓GSH Inhibit TGF-β1 ↓SOD	[32]
Fish oil Omega-3 supplementation	AST ↓ALT↓	[33]
Psolus phantasy	Anti-hepatic fibrosis	[34]
Celleporina secularism	Antitumor agents, Anti-hepatic fibrosis	[35]
Actinostola callosa	Anti-HIV, Anti-Cancer	[36]
Sea cucumber	CAT↑ SOD↑ MDA↓ TC↓ TG↓ HDL-C↑ Peptide, Antioxidant	[37]
Camel milk peptide	CAT ↑SOD ↑ mRNA expression, GSH-PX↑, GST↑	[38]
Bluefin leatherjacket (*Navodon septentrionalis*)	Antioxidant activity in three peptides	[39]
Blue mussel (Mytilus edulis)	PIIVYWK (1004.57 Da), TTANIEDRR (1074.54 Da), FSVVPSPK (860.09 Da) Antioxidant activity	[40]
Lactobacillus paracasei	TNF-α ↓, TGF-β1↓,	[41]
Lactobacillus casei	α-SMA↓	[42]
Weissella confuse	TNF-α ↓, TGF-β1↓,	[43]
Trichurus spiralis Hasselbr	ALT and AST ↓ Total protein↑	[44]
Buffalo skim milk	ALT and AST ↓ Total protein↑ ALP↓ Total Lipids↓ and Triglycerides↓	[45]
Snails (*C. mistrial*)	Antimicrobial, cytotoxic, anti-tumor and anti-inflammatory, Antileukemic, antineoplastic and antiviral properties	[46]
D-galactosamine (whey protein)	ALT and AST ↓ Total protein↑	[47]

Golden grey mullet protein (L. Murata)	Molecular weight 3–20 kDa contains glutamic acid (Glu) and glutamine (Gln), lysine (Lys), proline (Pro), leucine Leu) and glycine (Gly). ALT↓, AST↓, GLUCOSE↓, LDH↓, ALP↓, RBC Ht Hb CMV WBC Plt	[48]
Tenebrio molitor (novel peptide)	<1 kDa Protein show highest protective effect against H2O2-induced cytotoxicity in AML12 cells (mice hepatocyte). Catalase, β-actin antibody, and HO-1 antibodies for western blot. peptides identified with HPLC LC-MS, and LC-MS/MS.	[49]
Goat milk	AST↓ ALT ↓CYP2E1 ↓ TNF-α↓MDA↓, SOD, and GSH↓	[50][51][52]
Fish sardinelle (Sardinella aurita)	Protect hepatic and kidney function significantly	[53]
Soft coral *(Lobophytum crissum* and *Lobophytum duru*)	Hepatoprotective AST↓ ALT ↓	[54][55]
Snail (Bellamia bengalensis) flesh extract	ALT↓, AST↓, ϒGT↓, GOT, ACP↓, ALP↓, LDH↓, TG↓ TOTAL PROTEIN↑, bilirubin↓, LDH↓, MDA↓, SOD↑, GSH↓, CAT↑, GPx↓	[56]
Collagen Peptides from Cod Skin	ALT↓, AST↓, SOD↑, CAT↑, MDA↓	[57]
Red Sea sponge	ALT↓, AST↓, Polyphenolic compounds PDGF-Rβ signaling, Interfering NF-κB pathway, Interfering TGF-β pathway	[58][59]
Microalgae (Navicula incerta)	Peptides (NIPP-1 & NIPP-2), Interfering TGF-β pathway	[60]
Molluscs (Mytilus galloprovincialis)	Amino acids, Anti-inflammatory	
Eisenia bicyclis	Polyphenolic compound and hepatoprotective activity show Hepg2 cell line.	[61]
Marine Algae (*Pelvetia siliquosa*)	ALT↓, AST↓, GOT↓, SOD↑GSH-px↓	[62]
Krill Protein (*Euphausia superba*)	Molecular weight base fraction <1 kDa, 1–3 kDa, and >3 kDa. Downregulating TNF and interleukin-6 (IL-6) mRNA expression. SOD↑, CAT↑, GPX↓.Nrf2, HO-1 protein expression. Bcl-2↓, Bax↓.	[63][67]
Fungus, Cordyceps sp	Anti-fibrotic, Immunomodulatory, Anti-inflammatory Against thrombosis	[64][68]
Insect Extracts protein	Observed RT-PCR expression leptin, adiponectin, SREBP-1, adipocyte protein 2 (AP2), PACC, UCP2, Fit2/Fitm2, PPARϒ, Fsp27/ Cidec expression. TNF-α, IL-1β, IL-6.	[65][69]
Spider (*Nephila clavate*)	Expression of p-Smad 2/3, p-Smad3 and Smad3 Western blot analysis SMP30 in hepatocytes. Downregulation TGF-β/Smad.	[66][70]

2.3 Usage of different earthworm species in different traditional medicinal system

From the 10th to 18th centuries, *Lumbricus spp.*, an earthworm species, was used in the Levant region for treating ailments like haemorrhoids, earache, arthritis, and urinary tract obstructions [72]. In Nigeria, earthworms were used for therapeutic purposes, dietary consumption, and cultural and religious festivities. They were also used to treat guinea worm infections and infertility and provide prenatal and postnatal care for pregnant women [73]. Earthworms were also used in rituals to please deities and foster harmony within and outside communities. In Nepal, the Jirels have extensive knowledge of earthworms' therapeutic properties, using them to manage health conditions like measles, diarrhoea, jaundice, and pneumonia [74]. Traditional Chinese medicine (TCM) “Di Long” is made from extracts of the *Lumbricus rubellus* earthworm. It is used to treat a wide range of conditions, including seizures, fevers, rheumatoid arthritis, blood stasis syndromes, chronic bronchitis, asthma, psychosis, digestive tract ulcers, urticaria, burns, scalds, bladder calculi, urinating obstruction, cancer, and drooping upper eyelids [75]. Two types of Di Long are “Guang Di Long” and “Tu Di Long”. According to TCM “Di Long” is salty and chilly, connected with the bladder, liver, lung, and spleen meridians, possibly through draining liver heat, removing lung heat, and clearing collateral channel heat. It is rich in lumbrofebrine, lumbritin, terrestro-lumbrolysin, hypoxanthine, xanthine, adenine, guanine, choline, guanidine, ornithine, lysine, serine, proline, glycine, cystine, valine, phenylalanine, tryptophan, neutral lipids, cholesterol, free fatty acids, triglycerides, complex lipids, phosphatidylcholine, phosphatidyl ethanolamine, and phosphate. Earthworms have shown promise as sources for therapeutic extract, and their extract contains anti-oxidant enzymes such as glutathione, glutathione-related enzymes, and catalase [76].

Earthworm paste (EPA) from *Lampito mauritii* has been studied for its antiulceral and antioxidant effects compared to ranitidine. Balamurgan (2007) found that the earthworm

extract protects against paracetamol-induced liver injury by reducing enzymes. *Perionyx excavatus* powder, which is made from dried earthworms, helped protect the liver and fight free radicals in rats that had been feed alcohol too much, even though it increased lipid peroxidation and decreased antioxidant enzyme activity [77]. The total earthworm paste (TEP) of *Lampito mauritii* has been found to have anti-inflammatory effects on albino rats, with the highest activity observed at 160 mg/kg TEP in a carrageenan-induced edema model [78]. The pain-relieving activity of TEP was comparable to aspirin. The G-90 similar paste of the earthworm *Lampito Mouritii* showed similar anti-inflammatory properties to standard anti-inflammatory medications [79]. The whole tissue extracts of the earthworms *Allolobophora caliginosa* Savigny and *Pheretima hawayana* Rosa also showed anti-inflammatory, antipyretic, and antioxidant properties [80]. Hori *et al.* discovered antipyretic properties in earthworms, including *Lumbricus spencer* and *Perichaeta communishima*. These earthworm decoctions contain arachidonic and cis-5, 8, 11, 14, and 17-eicosapentaenoic fatty acids [81]. Balamurgan et al. found *Lampito Mauritii* reduced hyperpyrexia in rats, while Omar *et al*. found earthworm extracts reduced rectal temperature [79] [81]. *Eisenia foetida*, a species known for its hemolytic activity in coelomic fluid (CF), has been extensively studied due to its biochemical features. The cytotoxic impact of CF is linked to its hemolytic activity, with three of the seven hemolytic fractions obtained using electro-focusing showing cytotoxic effects [82]. A cytolytic protein, coelomic cytolytic factor-1 (CCF-1), was identified and isolated from the coelomic fluid of *Eisenia foetida*, contributing to almost 40% of the cytolytic activity [83]. The cytotoxic activity of *E. fetida* was attributed to the presence of two proteins, fetidins, with molecular weights of 40 and 45 kDa, which were activated by serine proteases in the coelomic fluid [83]. A protein with a molecular weight of 38.6 kDa was extracted from the earthworm coelomic fluid protein (ECFP) of *E. fetida* [82][84]. Despite its ultimate goal of eradicating cancer cells without

harming healthy tissue, cancer treatment currently lacks that ideal. Earthworm crude extract has been shown to kill cancer cells *in vitro* and suppress tumour growth *in vivo*. Earthworm proteases have improved efficacy of radiation and chemotherapy treatment [85][86]. Earthworms thrive in soil with abundant pathogens and are adapted to develop defence mechanisms against unfavourable conditions, allowing them to survive in such environments [87]. Humoral antibacterial activity [88][89] and strong cell-mediated defensive responses [90][88][91] have been widely examined in the earthworm *Eisenia foetida*. Natural inhibitors of *in vitro* bacterial growth have been found in the CF of annelids, and they include both gram-positive and gram-negative bacteria. Different antibacterial proteins (M.W. 45, 40, 20 kDa) were extracted from the CF of E. foetida [88][92]. Lassalle *et al.* (1988) discovered lysozyme protein, which enhances the antibacterial properties of CF. Injecting harmful bacteria into earthworms increases their antibacterial activity [93]. Both pathogenic strains were equally susceptible to this heightened activity [93]. Andrei et al. stated that two different proteins 45 kDa and 40 kDa were found to be primarily responsible for the antibacterial action of *Eisenia fetida* coelomic fluid [94]. Several studies in the field of modern medicine have shown that the CF of earthworms has high antibacterial action against various kinds of bacteria [88][87][94].

A new antibacterial peptide, lubricin-I, was isolated from *L.rubellus* coelomic fluid and inhibited bacteria growth without hemolysis [95][85]. Purified peptides, including PP-1 and 40-amino-acid-long "antibacterial tetradecapeptide," were stable for 180 days [85]. Dry earthworm powder showed high antibacterial action against *S. aureus, P. mirabilis*, and *P. aeruginosa* [77]. Verma and Verma (2012) identified and characterised an antibacterial peptide from Pheretima CF, a serine protease with wide antibacterial activity. The peptide was stable at various temperatures, pH, and inhibitors [96]. Earthworms have shown promise in treating various diseases, as antioxidants and also helped in regenerating amputated parts

[97][98][99][100]. A study on *Eisenia fetida's* glycolipoprotein tissue homogenate extract (G-90) showed it activates signal transduction pathways, increasing EGF and FGF activity in healthy skin and physiologically healing lesions and boosting growth factors by 10 and 5-fold, respectively [100]. In 2010, Matausic-pill *et al*. compared *Eisenia foetida* extract (G-90) to Panthenol-D, a routinely used wound treatment drug, on wound healing in an animal model. The study examined the molecular migratory pathways in Schwann cells for neuron regeneration produced by the earthworm *Pheretima aspergillum* (*E. Perrier*). The results showed that earthworms induce Schwann cell migration and up-regulate the production of PAs and MMP2/9. The MAPK pathways, ERK1/2, and p38 mediate this effect [101]. The study also examined the molecular pathways behind promoting neuron regeneration by *Pheretima aspergillum* extract using RSC96 cells. The results showed that administration of earthworm extract resulted in the phosphorylation of the insulin-like growth factor-I (IGF-I)-mediated phosphatidylinositol 3-kinase/serine-threonine kinase (PI3K/Akt) pathway, triggering an increase in protein expression of cell nuclear antigen (PCNA) in a time-dependent manner. The cell cycle research indicated that the G1 phase transitions into the S phase within 12–16 hours, while the S phase transitions into the G2 phase around 20 hours followed exposure to earthworm extract. The expression of cyclin D1, cyclin E, and cyclin A exhibits a time-dependent pattern of upregulation. The use of small interfering RNAs (siRNA) led to the effective suppression of PI3K, leading to a substantial decrease in the expression levels of the PI3K protein [101]. This resulted in a notable drop in the BCL-2 survival factor and a considerable impediment to actively dividing cells' transition from the G1 to S phase. The presence of earthworm extract enhanced the growth and viability of RSC96 cells via the activation of IGF-I signaling, with the PI3K protein playing a crucial role in mediating this process.

2.4 The rationale for the selection of earthworms as a natural hepatoprotective agent

Earthworms have a prehistoric relationship with humans, dating back to around 600 million years ago, when they evolved from aquatic polychaetes [102][103]. They are the first multicellular invertebrates to adopt a terrestrial lifestyle, but they are also amphibious. Earthworms are the dominant invertebrate species in temperate and tropical soils, and their soil-improvement potential has been recognised since prehistoric times [104][105][106]. Greek philosopher Aristotle called earthworms "The Intestine of Earth." Scientific observations on earthworms have been made by various scientists, with Charles Darwin recognising their role in soil waste breakdown [106][107]. Modern scientists agree that earthworms help preserve soil fertility. China, Japan, Korea, India, Cambodia, Myanmar (Burma), Vietnam, Iran, and the Middle East have utilised earthworms as food and medicine since ancient times (< 4000 years, 2600 B.C.). They may treat chronic cough, diphtheria, jaundice, rheumatic pains, TB, bronchitis, facial paralysis and impotence internally while cure wounds, persistent boils, piles, sore throats, and many more external treatments. Indigenous communities in the Indian subcontinent use earthworms for nutrition and healthcare [107]. Balavaidyas in north Kerala use earthworms and other animal products to create medications for children's ailments [108][109]. The raw Pheretima species is used as an antidote for snake and spider bites by 11 Naga tribal groups in Nagaland. The *Pheretima Posthuma* earthworm is used by Irular, Mudugar, and Kurumbar tribal communities in the Attappady hills of Kerala to treat wounds, chronic boils, piles, sore throats, chronic cough, diphtheria, jaundice, and paralysis [109][110]. Pakistani traditional medicine uses earthworms to treat wounds, chronic folds, piles, and sore throats [111]. 17th-century Irish scientist Robert Boyle suggested converting white wine-cleansed earthworm into powder, mixed with ambergris, for its fragrance and increased medicine efficacy [112]. This method is recommended for treating convulsions and treating piles with earthworm powder and hens-grease.

2.5 Pharmaceutical Importance

Earthworms are crucial in human health improvement, bioremediation, and environmental monitoring [113]. Research into vermiculture is growing, with applications in environmental protection and sustainable development. Earthworms have been ecosystem engineers for 600 million years, providing benefits in waste management, soil management, fertility improvement, and plant growth promotion [114]. Recent discoveries include their role in wastewater treatment, contaminated soil remediation, and potential use in modern medicine for human health protection. Composting and vermicomposting are pollutant-free bioconversion techniques for reusing organic waste, addressing waste management issues in municipal, agricultural, and industrial sectors [115][116].

2.6 Rationality behind taking medication with milk

Consuming medications with milk can help buffer the stomach's acidic environment, reduce irritation, and improve the absorption of fat-soluble drug like moieties. Lipophilic components of milk can also improve the absorption of vitamins and antibiotics [51]. Concurrent use of medications with milk can mask unpleasant tastes or odours, making the process more palatable for individuals, especially children. Additionally, milk provides essential nutrients like calcium and protein, making it an effective condiment/supplement option [51][52].

2.7 Bio-Nutrient value of earthworms

Earthworms, particularly Kurekure and White, are a popular food source in New Zealand due to their sweet residual flavour [117][118][119][120]. They have been used as dietary options for elderly individuals and even delivered as last meals for those in the latter stages of life [105, [121][122]. Earthworms have been used as medicine in indigenous populations across Australia, New Zealand, Japan, China, New Guinea, Africa, and South India

[123][124][125][126][127][128][129]. They contain a substantial protein content, making them a commonly consumed food source among many animal species. Earthworms fed on sterilised filter paper are used as animal and possibly human food in the United States and the United Kingdom [130][131]. A Venezuela tribe recommends to use earthworm as food to pregnant women and new mothers for first few months [132][133][134][135][136]. Earthworm contain calcium, iron, and other nutrients, and their chemical makeup is similar to that of vertebrates. Earthworms have a high concentration of essential vitamins and minerals, including iron and calcium, and 78–79 g/L free amino acids [137][138]. They also contain lysine, methionine, cysteine, and tyrosine [139], which are crucial components of human and animal feed.

2.8 Medicinal Value

Earthworms have been used for centuries in various regions, including ancient Burma and Laos, where they were used to treat smallpox patients. In Japan, earthworms produce four medicinal products: antibiose, aphrodisiacs, antipyretics, and antidotes [140]. In Indonesia, people use an indigenous traditional medicine called "Jamu" for treating dysmenorrhea and the after-natal care of pregnant women. Earthworms are also used to cure typhoid fever, breast tumours, nasal polyps, headaches, hypertension, and diuretics [141][142]. From the 1700s onwards, earthworms were commonly used in European medical practice, known for their diuretic, diaphoretic, and analgesic properties [143]. They were prescribed to treat apoplexy, convulsions, jaundice, dropsy, colic, gout, joint, ear, and tooth pains [144]. They were also prescribed against pneumonia and cystitis and used to treat snake and scorpion bites [145]. In China, earthworm medicine is known as "Jiryu" or "Jiru," with earthworm powder used to treat various medical conditions [146]. The earthworm (Di-Lung) is described as salty in taste, cold in property, efficacious in clearing the heart, invigorating blood circulation, dissolving stasis, opening up channels, curing stroke, hemiplegia, and infantile

convulsion. Earthworm preparation involves grinding dried earthworms, decoction, and ash. In Chinese medicine, earthworm tissues are used in ointments and extracts to treat various diseases [146][147]. Although few scientific studies have been conducted on the potential health benefits of earthworms, they have been used in various cultures worldwide for centuries [146]. Professor Shen Homgran's research in China in 1970 demonstrated the potential of earthworm formulations in treating cardiovascular problems [147][148][149][150]. He isolated and purified a potent fibrinolytic enzyme complex from the earthworm species *Lumbricus rubellus*, which contained six proteolytic enzymes collectively called lumbrokinase (LK) [150][151][152][153]. These enzymes showed significant efficacy in treating thrombosis and dissolving intravascular fibrin clots. This enzyme, a chemically based fibrinolytic medication, is cost-effective, convenient to store, and safe for oral use [152][154]. It is recommended for acute and chronic cardiovascular difficulties [153][154][155]. *Eisenia fetida* and *Eisenia Andrei* are two types of earthworms from which protein has been successfully isolated, purified, characterised, and crystallised [156][157]. The "wonder drug" is an enzyme complex called lumbrokinase, a serine protease, and an effective thrombolytic agent. It is safer, more stable, easier to store, and can be taken orally [158]. In experimental rabbits, monoamines were found to be responsible for the antipyretic effect of earthworm tissue extracts [159]. Inhibition of breast tumour development in mice by lubricin isolated from earthworms was established, suggesting the medicine may have some use for diabetic people [160]. Enzymes and other bioactive compounds derived from earthworms have anti-tumour and anti-cancer effects [161]. However, drugs made from earthworms are unavailable in India due to high import costs and regulatory hurdles. Very little research has been done on earthworms so far. However, several publications have already been published on therapeutic ideas for earthworm proteins or enzymes, which might play a special role in the future of new drug discovery.

2.8.1 Hepatotoxicity assessments and study model

Liver problems are a severe health risk in underdeveloped nations, resulting from poor food, alcohol abuse, cleanliness, uncontrolled drug use, and smoking. Non-inflammatory, inflammatory, and degenerative liver disorders exist. Due to hepatic insufficiency, increased plasma total cholesterol (LDL-C) and triacylglycerols (TGs) increase the risk of atherosclerosis and cardiovascular disease [161][162]. Liver diseases are clinical abnormalities or disorders that impede liver function. The primary forms of liver disorder are acute and chronic. Acute liver diseases are short-lived and begins quickly, whereas chronic liver disease lasts beyond six months. Chronic diseases leads to liver fibrosis and cirrhosis due to the periodic breakdown of liver parenchyma regeneration [164]. Significant liver inflammation, which can result from hepatotoxicity, a toxic effect from chemicals, is the root cause of chronic hepatitis, cirrhosis, and liver cancer. This can occur when medications are used excessively or at safe therapeutic levels. Chemicals such as nutritional supplements, alcohol, and medications can induce toxic hepatitis, with exposure lasting hours or days. Common household items, laboratory chemicals, industrial chemicals, natural compounds, and herbal medicines are some of the substances that can cause liver toxicity. Hepatotoxins, harmful chemicals, are linked to over 900 medications, leading to drug recalls. Subclinical liver damage often only appears on liver enzyme tests. About half of acute liver failure cases and 5% of hospital admissions are related to drug-induced liver disease [165]. Over 75% of patients with a distinctive medication response die or require liver transplants due to lipid peroxidation and other oxidative mechanisms. Liver damage reduces the body's antioxidant system, and environmental factors like X-rays, pollution, UV rays, and mitochondrial metabolism contribute to ROS production [166].Lipid peroxidation and covalent binding are common processes where free radicals cause liver injury or damage. Damage to membrane lipids, proteins, and nucleic acid is linked to age-related diseases like atherosclerosis,

diabetes, lung and kidney damage, cancer, and cardiovascular diseases [167][168]. Membrane lipid peroxidation disrupts membrane structure and function, which may harm the cell's ability to keep ion gradients and transport steady [169]. However, over time, excessive drug use and other substances might harm the liver [170]. Liver disease happens to us at different times. Most of the time, we cannot understand it. But when the body starts to deteriorate, if we take a test with the doctor's advice, we can understand that there is some kind of problem in the liver. By following the doctor's instructions, fatty liver or jaundice can slowly improve. But if they are hepatitis A, B, or C, or liver cancer, or steatosis, there is no primary treatment to cure them. Therefore, those scientifically involved in alleviating various diseases do not apply it directly to humans; artificially, different animals and newly discovered drugs are tested by producing toxicity on useful cell lines. Liver damage can be analysed using cell culture techniques in a laboratory setting to investigate the effects of toxins, drugs, or disease-related conditions on liver cells in a controlled environment. Common cell lines used include HepG2, Huh-7, and primary hepatocytes [171][172][173]. Liver cells are cultured in a sterile environment using specialised media and supplements. Chemical toxins, viral infections, oxidative stress, and inflammatory conditions can induce liver damage. Cell viability is assessed using various assays, while liver-specific enzymes are measured. Morphological changes, gene and protein expression analysis, functional assays, and inflammatory and oxidative stress markers are also used to assess liver cell functionality. Advanced techniques like 3D cell culture systems and microfluidic devices can provide more physiologically relevant models of liver damage and its progression [174]. Analysing liver damage in cell culture provides valuable insights into the mechanisms underlying liver injury and allows for the screening of potential therapeutic interventions. It is an important step in studying liver diseases and drug development, as it helps bridge the gap between basic research and clinical applications. Some such toxic mechanisms are given in the table below.

Table 2. Hepatotoxicity model

Treatment	Animal used	Dose and route	References
Carbon tetrachloride-induced hepatotoxicity	Male Sprague-Dawley rats	CCl_4 0.5 ml/kg i.p. for 3 days	[175]
	Male Sprague-Dawley rats	CCl_4 0.2 ml/100 g i.p. for 2 weeks	[176]
	Male Wistar rats	CCl_4 0.5 ml/kg i.p. twice a week for 4 weeks	[177]
	Male Wistar rats	CCl_4 0.125 ml/kg i.p. for 7 days	[178]
	Wistar strain albino rats	CCl_4 1.0 ml/kg i.p. after every 72 h for 10 days	[179]
	Male Wistar rats	CCl_4 2 ml/kg, s.c. at every 72 hours for 10 days	[180]
	Wistar rats Male/Female	Carbon tetra chloride gavage a dose of 1 ml/kg 5-6 days	[181]
Alcohol-induced hepatotoxicity	Male albino Wistar rat	Ethanol 7.9 g/kg p.o. daily for 45 days	[182]
	Wistar albino rats	Ethanol 2.0 ml/l00 g p.o. for 21 days	[183]
	Male albino Wistar rats	Ethanol 5 g/kg/day p.o. for 60 days	[184]
	Wistar female rats	Ethanol 3.76 gm/kg twice a day p.o. for 25 days	[185]
Radiation-induced hepatotoxicity	Male Wistar albino rats	Single-dose of whole-body gamma rays (6 Gy) for 15 consecutive days	[186]
	Male Sprague-Dawley rats	Rats exposed to 5 Gy of c-radiation for 2 days	[187]
	Male Wistar albino rats	Rats were irradiated at an acute single-dose	[188]
	Male Wister albino rats	level of 3 or 6 Gy for 7 days	[189]
Metal-induced hepatotoxicity	Male Wistar rats	Mercury chloride (HgCl2) (80 mg/l) as drinking water for 4 weeks	[190]
	Male albino rats (Male/female rats)	5 mg/kg s/c injection of mercury (Hg) , Mercuric Chloride on the 7th Day	[190][191]
Drug	Male Wistar rats	500 mg/kg D-galactosamine over a priod of one to 3 months	[192]
	Male Wistar rats	Thioacetamide 400 mg/kg i.p. for 2 weeks	[193]

	Male Wistar rats	Erythromycin isolate 800 mg/kg/d for 15 days	[194]
	Rat liver cell damage and biliary cell damage with cholestasis at 24 h	Alpha-Naphthyl isothiocyanate 75 mg/kg, intraperitoneal	[195]
	Wistar albino Rats	Tamoxifen An IP dose of 45 mg/kg/d of tamoxifen citrate in 0.1 mL dimethyl sulfoxide and normal saline for 6 days	[171]
	Wistar albino Rats	Paracetamol 300 mg/kg 7 days	[196]
	Wistar albino Rats	INH Dose: 200 or 400 mg/kg/day Administration: Gavage daily for One week Dose: INH 75 mg/kg/day and RIF 150 mg/kg/day Administration: Gavage daily for one week	[197]
In vitro	Liver cell line	Exposure Time	
	HepaRG/hiPSCderived HLC	24 h/7 days single /72 h single	[198][172]
	PHH	14 days repeated	[173]
	HepG2/ PHH-fibroblast	24 h single OR 6 days /9 days repeated	[198]
	Fresh hepatocytes	Quick and cheap tests	[172]
	Primary hepatocyte culture	High control of variables; reproducible	[173]
	Immortalized cell lines (HepG2, HUH7, HepRG)	change media 14-15 days	[173]
Ex vivo	Precise liver cuts	have in common with the in vivo environment	[172]
	Isolated perfused liver	support limited animal and human tissue growth properly.	[173]

2.8.2 Molecular and Cellular Biological Markers

The liver releases a variety of proteins when there are issues with the liver. Among them are special proteins known as markers, whose increase and decrease indicate severe liver problems. A very useful model can be used to determine the dose of a drug for protection. Some markers that predispose to liver damage include pro-inflammatory, special cell

degeneration, and mitochondrial damage. Recently, several studies have shown that the expression of different protein types dictates the liver cells' regeneration pathway. Tumour necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine important in mediating the immune response. TNF-α exhibits many actions inside the liver, including facilitating inflammatory processes and its involvement in the pathogenesis of liver damage in hepatitis and non-alcoholic fatty liver disease (NAFLD) [199]. The protein kinase AKT1, also known as Protein Kinase B, plays a crucial role in several physiological functions, such as cell survival and proliferation. Growth factors and cytokines facilitate the activation of this process, and it is associated with the promotion of liver cell survival and proliferation [200]. Bax is a protein with pro-apoptotic properties, serving as a regulator of the process of programmed cell death, also known as apoptosis. The equilibrium between pro-apoptotic proteins, such as Bax, and anti-apoptotic proteins may influence the survival of liver cells. The protein α-SMA, also known as Alpha-Smooth Muscle Actin, is present in smooth muscle cells and myofibroblasts [201]. Elevated α-SMA expression in the liver is correlated with liver fibrosis, a pathological condition characterised by the formation of scar tissue in the liver due to prolonged hepatic damage [202]. Nrf2, also known as nuclear factor erythroid 2-related factor 2, is a transcription factor responsible for regulating antioxidant and detoxifying enzyme expression. The activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) has been shown to protect hepatic cells against the detrimental effects of oxidative stress and subsequent damage [203]. Interleukin-6 (IL-6) is a cytokine crucial in mediating inflammatory processes and immunological responses. Elevated levels of interleukin-6 (IL-6) in the liver can lead to inflammation and damage inside the hepatic organ. Interleukin-4 (IL4) is a cytokine involved in regulating the immune system [204]. Within the hepatic organ, it can regulate the immune response and impact the equilibrium between inflammatory processes and tissue restoration. Stat3, also known as Signal Transducer and Activator of

Transcription 3, is a transcription factor activated by a range of cytokines, including interleukin-6 (IL-6). It regulates gene expression in response to cytokine signalling and can impact liver inflammation and regeneration [204].

2.8.3 Plant-source-based hepatoprotective activity

Plant sources with hepatoprotective properties can help protect the liver from damage and support its function. Some of these plant sources include milk thistle, turmeric, artichoke, dandelions, Schisandra, ginger, licorice, green tea, aloe vera, and burdock [205]. Milk thistle contains silymarin, an antioxidant and anti-inflammatory compound known for its hepatoprotective effects. Turmeric, with its active compound curcumin, has anti-inflammatory and antioxidant properties, aiding in liver disease treatment [206]. Artichoke leaf extract has hepatoprotective effects due to its antioxidant properties. Dandelion root and leaf extracts have been studied for their potential to support liver function and protect against liver damage [207]. Schisandra berries contain compounds that may protect the liver from damage and improve its function. Ginger, with its anti-inflammatory and antioxidant properties, may help protect the liver from toxins and oxidative stress. Licorice root has hepatoprotective properties, aiding in liver detoxification and inflammation [208]. Green tea, rich in antioxidants, may have a protective effect on the liver and help with liver diseases. Green tea contains six primary catechin compounds with detoxification and hepatoprotective properties. Emblica officinalis fruit extract has antidiabetic and liver-specific enzyme alanine transaminase activity-lowering activity [209]. Terminalia arjuna has medicinal activity, down-regulating LDL cholesterol levels and reducing sugar levels, and is effective in regulating hepatoprotective activity. Silybum marianum seed extract contains a flavonolignan complex, fatty acids, and linoleic acid, providing relief from hangovers for heavy drinkers [210]. Azadirachta indica leaf and bark have bitter and astringent properties, healing wounds and regulating severe conditions like cough, vomiting, skin diseases, excessive thirst, diabetes,

and jaundice. *Ocimum sanctum* (Tulsi) has potent COX-2 inhibitors, painkillers, and antihyperlipidemic, cardioprotective, and hepatoprotective activity. Aloe vera gel promotes liver health and protects against liver damage [211].

2.8.4 Hepatotoxic agents

Numerous xenobiotics, including chemicals, pharmaceuticals, household substances, herbal products, and environmental agents, have been well-recognised for their ability to produce hepatotoxicity. The centrilobular (zone-3) hepatocytes are the primary locations of hemoprotein P450 enzyme activity, rendering them particularly susceptible to xenobiotic-induced liver damage, which is of great significance. The compounds mentioned in the study conducted by Hernandez-Aquino *et al*. (2017) [175][176][178][212] include carbon tetrachloride (CCl_4), N-nitroso diethylamine, acetylaminofluorene, galactosamine, d-galactosamine/ lipolysaccharide, TAA, antitubercular medicines, paracetamol, and arsenic.

2.8.4.1 CCl_4-induced hepatotoxicity model

Carbon tetrachloride (CCl_4) is a potent hepatotoxin that induces liver necrosis in experimental animals. Its metabolism by cytochrome P450 enzymes produces reactive trichloromethyl peroxy free radicals, leading to lipid peroxidation and liver cell necrosis. CCl_4 at a toxic dose of 0.1-3 ml/kg, causes centrilobular necrosis and lipid alterations within 24 hours [213].

2.8.4.2 Paracetamol-induced hepatotoxicity model

High dosages of paracetamol (PCM), a common painkiller and antipyretic, may cause severe liver damage. PCM injection produces centrilobular hepatocyte necrosis with nuclear pyknosis eosinophilic cytoplasm and extensive hepatic lesions. N-acetyl-P benzoquinoneimine, an oxidative product of PCM, covalently binds to protein sulphydryl groups, degrading lipid peroxidizing glutathione and causing liver cell necrosis. The oral PCM dose is 1-2gm/kg [174][2

Aim and objective of the study

Liver disease is one of the most common lifestyle diseases these days, affecting one in every three people. It is one of the greatest health challenges of the 21st century. Although this condition is common, easily recognized, yet no proper treatment unveiled till now. The current research aim of the work entitled "The Natural Remedies of Hepatic Diseases" was used as a hepatoprotective natural product. In order to conduct a research on natural remedies for hepatic diseases, the following research objectives are given:

Characterization of earthworm species and previous traditional reports

Identifying species and identifying specific bio extracts associated with hepatoprotection, traditionally used in the past, allows the development therapeutic aid for liver diseases.

Evaluate Safety and Tolerance acute and sub-acute toxicity study

To evaluate the safety and tolerance of the earthworm extract, by conducting toxicity tests, confirming that the treatments does not produce lethality and any hepatotoxicity by organ histopathology in animal model.

Optimize Dosage and Administration

To develop the most effective dose and delivery schedule for earthworm protein treatments in order to maximise their hepatoprotective advantages while minimising any possible adverse effects.

Explore Synergistic Effects

To examine the possible synergistic effects that may occur when combining various natural agent with conventional treatments to get improved therapeutic results.

Assess Hepatoprotective Effects

Assess hepatoprotective benefits of earthworm protein powder utilising in vitro and/or in vivo research, focusing on liver health biomarkers.

Compare Efficacy with Standard Treatments

Assess the effectiveness of the observed natural remedies in treating hepatic injuries by comparing them to treatment regimen, using suitable experimental models.

Determine Mechanisms of Action:

Identify the modes of action such as anti-inflammatory, antioxidant, and anti-fibrotic pathways by which the bioactive chemicals that have been discovered impart their hepatoprotective benefits.

Identify Bioactive hepatoprtective component:

Investigate bioactive components in natural sources that may protect the liver.

Investigate Long-Term Effects:

To investigate the potential of natural therapies for liver health in the long term, checking specifically at their capability to prevent the progression of injury and keep hepatoprotection.

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CHAPTER -III

The Protective Action of Earthworm Protein Powder/ MWCO 50 kDa Fraction on Experimental Hepatic Damage: An In-vivo /In-vitro Approach Involving Inflammatory Tnf-α and Il-6

Abstract

This study was to investigate *invivo* and *invitro* hepatoprotective role of Indian earthworm (*Pheretima posthuma)* protein powder (EP) in rats against paracetamol induced liver damage. Briefly, the animals were divided into 4 groups (n=6) of wister albino male rats. Normal group, 1 ml of 20% Tween 80 (vehicle) was administered orally for 2 days in a week for 14 days. Paracetamol-treated group, paracetamol (750 mg/kg p.o.) suspension was administered orally twice a week for 14 days. Paracetamol-EP treated group, paracetamol suspension was administered concurrently with EP (266 mg/kg b.w), orally twice a week for 14 days. Paracetamol and silymarin treated group, paracetamol suspension was administered concurrently with silymarin (100 mg/kg p.o.) orally twice a week for 14 days. Paracetamol–treated animals exhibited a significant ($P<0.05$) elevation of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, LPO or MDA, total bilirubin, mRNA expressions of TNF- α and IL-6 while the level of CAT, SOD, GSH significantly ($P<0.05$) decreased in the paracetamol group and was later restored in paracetamol-EP treated group. Similarly, the separated MWCO 50 KDa protein obtained from EP (lyophilized) using Sephadex-G75 showed significant ($P<0.05$) reduction of AST and ALT, ALP and bilirubin in primary hepatocyte culture supernatant and increased percentage viability. The histological examination of paracetamol group showed disruption of the liver histoarchitecture and its restoration was found in the paracetamol-EP group. The *invivo* study, showed that the animals in the paracetamol-EP and the paracetamol-silymarin groups showed restoration of biochemical parameters, oxidative stress markers, mRNA expressions and histopathological findings. The acute liver damage due to paracetamol is attributed to the oxidative stress in the animal model. EP decreased oxidative stress as well as inflammation and slowed down the liver damage progression caused by paracetamol. EP (*Pheretima posthuma*) activities involving cytokine expressions e.g., TNF-α and IL-6 using lower molecular weight protein fraction from the dialysate i.e., MWCO 50 kDa and below protein fractions is of scientific importance with correlation to real time use.

Keywords: Paracetamol, Regeneration, Earthworm protein, Antioxidant and Hepatoprotective, SDS-PAGE.

3. INTRODUCTION

Liver, is responsible for equilibrium, metabolism, and excretion of the human body [1] and has the onus to detoxify xenobiotics and chemotherapeutic drugs [2]. Toxic chemical and drug-induced liver damage is known to be intimately related to generation of oxidative stress of free radicals [3],[4]. Drugs and chemicals namely - arsenic, Diclofenac, Carbon tetrachloride, Rifampicin and acetaminophen is well documented to induce hepatic injury in similar fashion [4]. Although, liver is a highly regenerative organ, hepatotoxicity often leads to severe clinical complications which may range from simple digestive disorder to even metabolic failure leading to death [5].Thus, acute liver failure in a wide variety of age group is an extremely severe clinical emergency with fewer medicinal and surgical options [6]. Paracetamol or Acetaminophen is a very safe OTC antipyretic and analgesic drug. However, overdose of this drug may cause centrizonal haemorrhagic hepatic necrosis. According to reports, the indiscriminate use of paracetamol in the United States is responsible for about 50% of overdose-related acute liver failure and nearly 20% of liver transplant cases [7]. Following oral administration of paracetamol, two-thirds of the drug is metabolised via glucuronidation, with the remaining metabolised primarily in the liver via sulphation. The kidney excretes the water-soluble metabolites of paracetamol that are generated during the metabolic pathways [8]. In paracetamol overdose intrinsic glutathione depletion results in accumulation of reactive metabolite N-acetyl p-benzoquinonimine (NAPQI). NAPQI binds covalently to cell-based macromolecules, causing cell damage [9]. It impairs mitochondrial function, produces reactive oxygen species (ROS), and causes oxidative stress. The formation of several cytokines and chemokines, such as TNF-α, IL-1, IL-6, IL-11, IL-10, MCP-1, MIP-1α, MIP-3A, alpha-SMA, Bax or Bcl-2 imbalance, Timp1, MIP-2, chronic wound healing agent VEGF, and IL-8, are associated with higher doses of acetaminophen [10]. After being activated, Kuppfer cells release chemokines that entice monocytes and neutrophils, as well as cytokines that amplify inflammation and predispose to hepatic damage, [8] eventually causing elevation of various plasma markers like ALT, AST & ALP in both human and laboratory rats. Silymarin (SILY.), a natural polyphenolic component is widely used as a clinical hepatoprotective agent [11]. Earthworms, due to their high nutritional content, have long been used against various diseases. Traditional Chinese medicine also claims such beneficial effect [12]. “Compendium of Materia Medica” also claims that over 40 human treatments are associated with the use of earthworms in china. Previous study on Earthworm demonstrates its antipyretic, anti-hypertensive and anti-microbial activity [13,[14],[15]. The

traditional use of Indian Earthworm or *Pheretima posthuma* [16] family Megascolecidae also finds mention in the Indian traditional medical system. It is octothecal with 4 pairs of spermathecal pores which are minute and superficially present on the posterior margins [17]. The pores are present at the midpoint of the translucent grayish area which is transversely elliptical. The Ventrally located seta is present on the clitellar segments. It is 60-140 mm in length with 4-8 mm in diameter.It is widely distributed in India, Pakistan and Southeast Asia. In India, it is also found in Dattapakur North 24 Parganas, a district of West Bengal. It has been observed that the locals of North 24 Parganas (Dattapakur) have long been using earthworm to treat patients with acute liver failure. These observations lead to the scientifically exploration for any probable therapeutic activity of this Earthworm protein powder against experimentally induced liver damage. Despite existing scientific claims for hepato-protective efficacy of Earthworm extract (*Lampito mauritii*, Kinberg) against paracetamol induced liver damage [14], we took up this study since our earthworm belongs to other genus and species as that of previous reports. Besides confirming earlier claims of in vivo hepatoprotection against paracetamol-induced liver damage by EP, we also tried to explore for probable involvement of cardinal inflammatory markers e.g., TNF-α and IL-6 in the mentioned protective pharmacology of EP. Our other objective of study was to isolate a low molecular weight EP fraction (we selected MWCO 50 KD) and assess its in vitro effectiveness in restoring altered clinical biochemical hepatic parameters induced by paracetamol- in primary hepatocyte culture. Biomarkers (both invivo and invitro), anti-oxidative enzyme activities, inflammatory cytokines, and histological examination were used to assess the extent of hepatotoxicity and hepatoprotective effect of this traditional product [14][12].

3.1. Material

Indian Earthworm was purchased from Lila agrotech Pvt Ltd, Madhyamgram, Paracetamol (sigma-aldrich, catalog no. A7085) and Silymarin was obtained (sigma-aldrich catalog no. S0292), Aspartate aminotransferase (AST) kit (coral clinical system, catatalogue no 1102200025), Alanine aminotransferase (ALT) kit (coral clinical system, catalog no 1102200025), Alkaline phosphatase (ALP) kit (coral clinical system, catalogue no.1101030015), Bilirubin kit (coral clinical system catalogue no.1101050035), Catalase Assay Kit (Sigma-Aldrich, Catalogue Number CAT100), Glutathione (GSH) Assay Kit

(Sigma-Aldrich, Catalogue Number CS0260), Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, Catalog Number MAK085), Superoxide Dismutase (SOD) Activity Assay Kit (Sigma-Aldrich, Catalogue Number CS0009), Penicillin-Streptomycin (Sigma-Aldrich, catalogue number 15140122), Fetal Bovine Serum (Thermofisher scientific, Catalogue number 26140087). SpectraMax (M5 Series Multi-Mode Microplate Readers), Respons 910(autoanalyzer), Thermo cycler (MJ research PTC200), Collagenase II (sigma-Aldrich,Catalogue number 1148090). Sephadex G-75 (Sigma-Aldrich, Catalogue number GE17004201), Vivaspin® 500 Centrifugal Concentrator (Sartorius), MWCO 50 kDa Polyethersulfone (merck,cat.no. GE28-9322-36), MTT solution (Thermofisher, Catalogue number M6494), William's E medium (Thermofisher, Catalogue no. 12551032), Acrylamide extrapure (SRL, India, Catalogue number 22794), bis acrylamide (SRL, India catalogue. no.67320), Coomassie brilliant blue R 250 (SRL, India, catalogue no.3567811), Ammonium persulfate (sigma-aldrich, catalogue number A3678). N, N,N′, N′-Tetramethyl ethylenediamine (TEMED) (Sigma-Aldrich, catalog number T22500), Minive vertical electrophoresis system (Amersham Biosciences corp. Amersham, United Kingdom), ImageQuant LAS 500 (GE Healthcare Life Sciences), Takara premixed protein marker (catalog no. 3597A), micro tube pump MP-3N (EYELA, Tokyo, Rikakikai co., LTD), Sample protector for RNA/DNA (Takara, Japan Code No. 9750), High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, India catalog no. 4368814), 1kb DNA Step Ladder (GCC biotech catalog no. G4669), TAE Buffer 10 x (hi-Grade) (Thermo Fisher Scientific, India, catalog number AM9869) and High gel agarose New, molecular biology grade (DNA/RNA) (GCC biotech, India. catalog no. G4652).

3.2. Method

3.2.2. Animals And Husbandry

The animal studies were conducted according to the established methodology set by the Committee for the Monitoring and Control Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The ARRIVE criteria and the National Institute of Health Guide for the Care and Use of Laboratory Animals carried out all animal studies. The Institutional Animal Ethics Committee (IAEC), Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India, authorized the experimental procedures vide approval number or ref no. AEC/PHARM/1702/3/2017. The rats were raised in a pathogen-free environment and could freely access food and water ad libitum. The room temperature was 22±2 °C, with a 12-hour dark/light period.

3.2.3. Preparation of Indian earthworm protein powder

Two hundred mature earthworms (average body weight 800 mg) were kept at room temperature in water for 4 hours until their digestive systems were cleansed. We only preserved the center section for use and removed the head and rear portion of it. The center portion of earthworm tissue weighing 40 g was mashed in 40 mL of chloroform with methanol solution (1:1 ratio) and stored at 4 °C for 4 hours. Then, 100 mL of distilled water was added. After centrifuging the mixture for 20 minutes at 4000 rpm, it separated into three distinct layers. The top layer of water/methanol was pipetted off and dried on a rotavapor till no methanol appeared. The extracted protein was then lyophilized (earthworm protein powder or EP), and the total protein content was determined using the Lowry technique [18],[19].

3.2.4. Animal Allocations and Treatment

3.2.4.1. Hepatoprotective effect of EP in the in-vivo experiment

A Total of 24 albino Wister rats (male rats 150-180 g) were separated into four groups (n=6). The animals of the normal group were administered 1 ml of 20% Tween 80 as a vehicle orally

twice a week. Paracetamol-treated group animals were administered with Paracetamol suspension in a high dose of 750 mg/kg b.w. Twice a week for 14 days. The animals of paracetamol and EP-treated group were administered with EP powder suspension in a dose of 266 mg/kg b.w concurrently with 750 mg/kg b.w paracetamol orally, twice a week for 14 days [20-22] while the animals of paracetamol and silymarin (reference drug)-treated group were administered with 750 mg/kg b.w paracetamol and silymarin (100 mg/kg p.o.) suspension concurrently orally, twice a week for 14 days. All the animals were provided with ad libitum food and saline water during the study.

3.2.4.2. Preparation of serum and tissue of the liver:

The rats were euthanized using pentobarbital (barbiturate) overdose and administered intraperitoneally, as per standard guidelines of the Institutional Animal Ethics Committee (IAEC), 24 hours after the final treatment. Blood was drawn from the Retro-orbital plexus and allowed to stand for 10 minutes before being centrifuged for 15 minutes at 4000 rpm centrifuge in a 40 C. The serum was collected and utilized for liver function tests. The liver was separated and divided into pieces for antioxidant tests, and the other part was kept for PCR test with a sample protector for RNA/DNA; one part of the hepatic tissue was homogenized according to the supplied kit, while the other part was preserved in 10% formaldehyde with distilled water preparation for histopathological inspection [23].

3.2.4.3. Activity of liver enzymes in the blood:

The liver function test marker enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and Total bilirubin were measured using a commercial kit.

3.2.4.4. Assay of Oxidative stress markers in liver tissue

The liver tissue oxidative stress marker, Superoxide Dismutase (SOD), Catalase (Cat) Activity, Lipid peroxidation or MDA assay, and Glutathione (GSH) activity was measured using commercial kits.

3.3.9.4. Estimation of Superoxide dismutase (SOD)

Principle: Superoxide dismutase molecule catalysis is the breakdown of superoxide-free radical (O2-) as per the following reaction [11]:

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

Reagents:

1. Sodium Pyrophosphate Buffer (0.052M): 2.319512 gm mixed in 100ml DH2O.
2. Phenazine methosulphate (PMS) (180 µM): 0.001 gm mixed with 20 ml DH2O.
3. Nitrobluetetrazolium (NBT) (300µM): 0.004gm mixed with 20ml DH2O.
4. Nicotinamide Adenosine Dihydride (NADH) (780µM): 0.006 gm mixed with 10 ml DH2O.

Note: PMS and NBT were prepared and kept at 40℃ in the dark.

Procedure:

This test was evaluated in two steps: -

Step-I:

1 ml of hemolysate was mixed with 250 µl of Ethanol and 150 µl of Chloroform, and the mixture was shaken well/vortexed and centrifuged at 2500 rpm for 15 min at 4ºC. Then 200 µl supernatant [Peri-mitochondrial fragment (PMF)][8][11][3] was removed and kept for the next step.

Step-I:

The violet color appeared in all mixtures of test and control after vortexing and incubating them at 370C for 90 seconds, after that, 1 ml of Acetic Acid was added to stop the reaction and O. D. was taken at 560 nm after 10 min[11].

Calculation:

$$\frac{100 \times A \text{ (absorbance of sample)}}{\text{Control}} = B$$

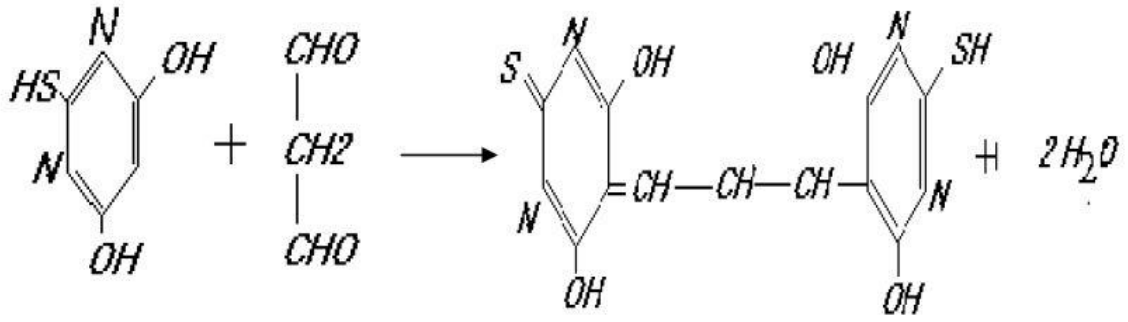
$$100 - B = C \text{ (\% inhibition)}$$

$$\frac{C}{50} = D$$

$$\text{SOD Units / min / mg protein} = \frac{D}{0.66 \times 0.150 \times \text{mg P (Protein in mg/ml)}}$$

3.3.9.5. Estimation of Thiobarbituric Acid Reactive Substance

The reactions involved and the procedure for the determination of Thiobarbituric acid reactive substance (TBARS) are in detail as follows [11]:



TBA **MDA** **COLOURED PRODUCT**

Reagent:

TBA Reagent: 25 mL

Standard: 50 µL 6 M MDA

10% Trichloroacetic acid (TCA): 25 Ml

Sample Preparation

Samples can be frozen at -80°C (stable for one month) if not assayed immediately. Urine samples are stable at 25°C or below for at least three days. Urine and saliva samples can be assayed directly (*n* = 1). The following samples need to be deproteinated before assay:

1. Transfer 100 µL of each sample into a labeled 1.5-mL tube for serum and plasma. For tissue samples, weigh ~20 mg into 200 µL ice-cold phosphate-buffered saline (PBS). Homogenize tissue by brief sonication (e.g., 20 seconds) on ice. If desired, remove 20 µL aliquot for protein

analysis. Place 100 µL tissue lysate into a labeled 1.5 mL microcentrifuge tube. For cells, harvest 5×10^6 cells in 200 µL ice-cold PBS and sonicate to disrupt cells. If desired, remove 20 µL aliquot for protein analysis. Place 100 µL cell lysate into a labeled 1.5 mL microcentrifuge tube[8].

2. Add 200 µL ice-cold 10% TCA to the 100 µL of each sample. Incubate for 5 minutes on ice.

3. Centrifuge 5 min at 14,000 rpm in an Eppendorf Centrifuge. Transfer 200 µL of each clear supernatant into a new labeled tube. The dilution factor for these pretreated samples is $n = 3$.

3.3.9.6. Colorimetric Assay Procedure

Set up a water bath or heat block and adjust the temperature to 100°C. Equilibrate all components to room temperature.

1. ***Standards***. First, briefly centrifuge the Standard tube to pellet any MDA stuck in the cap or on the sides of the tube. Then, mix 4 µL of the 6 M MDA with 2396 µL dH2O (final 10 mM MDA). Next, prepare 30 µM MDA by mixing 3 µL of the 10 mM MDA with 997 µL dH2O. Dilute standards as shown in the Table:

No	30 µM MDA + H_2O	Vol (µL)	MDA (µM)
1	300 µL + 0 µL	300	30.0
2	180 µL + 120 µL	300	18.0
3	90 µL + 210 µL	300	9.0
4	0 µL + 300 µL	300	0.0

Transfer 200 µL of each standard into separate, labelled 1.5-mL screw cap tubes. *Samples*. Transfer 200 µL of each sample into separate tubes.

2. *Color reaction.* To each of the standards and samples, add 200 µL TBA Reagent. Vortex tubes to mix and incubate at 100°C for 60 min. Cool down tubes to room temperature. Vortex and briefly centrifuge tubes.

3. Load 100 µL in duplicate from each tube to wells of a clear flat-bottom 96-well plate. Read OD at 535 nm (525 to 545 nm). The pink color (chromogen) so formed was read at 525 nm wavelength[3][9] [21].

Calculation

$$[\text{TBARS}] = \frac{R_{\text{Sample}} - R_{\text{Blank}}}{\text{Slope } (\mu M^{-1})} \times n \quad (\mu M \text{ MDA equivalents})$$

3.3.9.7. Estimation of Total Protein by Lowry Method

The Folin-Ciocalteau reagent reacts with protein to form a colored complex. This color is caused by the reaction of alkaline copper with the protein and the reduction of phosphomolybdate by the protein's tyrosine and tryptophan. The color intensity is proportional to the number of aromatic amino acids present [19[20][21].

Reagents:

(1) Na_2CO_3 (4%): 0.6 gm in 15 ml of DH_2O.

(2) $CuSO_4$.5H2O (1%): 0.01 gm in 1 ml of DH_2O.

(3) Na -K-tartarate (1%): 0.01 gm in 1 ml of DH_2O.

(4) Alkaline Copper Sulphate Solution: For 13 ml, take 250 µl of each $CuSO_4.5H_2O$ and Na -K-tartarate and 12.5 ml of Na_2CO_3.

(5) NaOH (0.1 N): 0.3 gm mixed in 75 ml of DH2O.

(6) Folin – Ciocalteu Reagent (2N): It was prepared from a commercially available solution by diluting 1 ml of folin's reagent with 2 ml of DH2O.

(7) Bovine Serum Albumin as Protein Standard (1mg/ml): 1 mg mixed in 1 ml of DH_2O.

Note: FCR reagent was made in the dark and at the time of the experiment. Reagents 1, 2 and 3 were prepared fresh [18].

Calculation:

Protein concentration (mg/ml) was evaluated by two steps:-

Step-I:

$$\text{Conc. of protein } (\mu g) = \frac{\text{Conc. of respective std. X Mean O.D. of sample}}{\text{O.D. of respective std.}}$$

Step-II:

$$\text{Conc. of protein (mg/ml)} = \frac{\text{Conc. of protein in } \mu g \text{ X } 1000 \text{ (as } \mu l)}{10 \text{ (Dilution factor)}}$$

Dilution factor: Sample was tested at 10 µl conc. that's why factor "10" was used in denominator.

3.3.9.8. Bradford protein assay:

The Bradford assay is a common laboratory technique used to determine the concentration of proteins in a solution quantitatively. It relies on the interaction between proteins and a special dye, Coomassie Brilliant Blue G-250, which changes color based on the protein concentration.

After performing the Bradford assay for protein samples, measure the absorbance of the samples at the same wavelength used for the standards (usually around 595 nm)[18][19].

Procedure

Measure the absorbance of a series of standard solutions with known protein concentrations (e.g., Bovine Serum Albumin or BSA) using the same Bradford assay procedure you used for your samples. Typically, you create a series of standard solutions with known concentrations (e.g., 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL)[19] [20].

Calculation

Concentration (mg/mL) = (Absorbance of Sample - Y-intercept) / Slope

3.3.9.9. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS PAGE):

SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) is a widely used laboratory technique for separating proteins based on their molecular weight. Here's a general procedure for performing SDS-PAGE [21]:

Stock Solution	Resolve Gel	Stacking Gel
30% Polyacrylamide Acrylamide 37.5g Bis-Acrylamide 1g Purified water makes up to 125 ml	Gel percentage 12% Total volume 10 ml	Gel percentage 4% Total volume 5 ml
1.5M Tris-HCL (pH 8.8) Tris 18.15g HCL make pH 8.8 Purified water up to 100 ml	**Calculation:** Purified water 3.300 ml 30% polyacrylamide 4.00 ml	**Calculation:** Purified water 2.978 ml 30% polyacrylamide 0.667 ml
0.5M Tris-HCL (pH 6.8) Tris 18.15g HCL make pH 6.8 Purified water up to 100 ml	1.5 M Tris-HCL (Ph 8.8) 2.500 ml	0.5 M Tris-HCL (Ph 8.8) 1.250 ml
10% SDS SDS 10g Purified water up to 100 ml	10% SDS 0.100 ml TEMED 4.000 µl	10% SDS 0.050 ml TEMED 5.000 µl
10 % Ammonium persulfate Ammonium persulfate 100 mg Purified water 1ml	10% ammonium persulfate 0.100 µl	10% ammonium persulfate 0.050 µl

Procedure:

1. **Prepare the Gel:**
    - Assemble the SDS-PAGE gel electrophoresis apparatus according to the manufacturer's instructions.
    - Prepare the resolving gel and stacking gel solutions according to your specific experimental requirements. These gels are typically made from acrylamide and bisacrylamide. Common percentages for the resolving gel are 10-15%, while the stacking gel is usually 4%.

2. **Load the Gel:**
    - Load the gel-loading buffer into your protein samples. The gel-loading buffer typically contains SDS, reducing agent (β-mercaptoethanol or DTT), and bromophenol blue.
    - Boil the samples in a water bath for a few minutes to denature the proteins and break disulfide bonds.
3. **Load the Samples:**

    Load the prepared protein samples and molecular weight standards into the wells of the stacking gel.
4. **Formula for stain:**
    - When Coomassie tablet/powder is used in staining for 100 ml
    - Methanol: Acetic acid: Water =30 :10 : 60
5. **Formula for destaining**: for 100 ml
    - Methanol: Acetic Acid: water =25 :10 :65

- If use Coomassie Brilliant Blue, destain the gel with a destaining solution. The protein bands will become visible as the background becomes clear.
- Document the gel by taking a picture or scanning it.

6. **Analyze the Results:**

- Analyze the protein bands in the gel. The estimate the molecular weight of unknown proteins by comparing them to the molecular weight standards. Densitometry or image analysis software can help quantify the band intensities.

7. **Optional Western Blotting:**

- If need to perform Western blotting or transfer proteins to a membrane for further analysis, this is typically done after SDS-PAGE.

3.4. Isolation of RNA

100 mg of hepatic tissue samples were homogenized from treated and untreated rats immediately after resection in a guanidinium-based denaturation solution. The RNA kit (Trizinxp RNA Isolation Kit) was isolated from the lysates according to the manufacturer's instructions. DNase I was used further to clean the RNAs of any remaining DNA contamination. A UV Spectrophotometer (Perkin-Elmer) was used to measure the absorbance of each RNA isolate at 260 and 280 nm. The concentration of RNA per sample was calculated using absorbances. According to the primer design, reverse transcription polymerase chain reaction analysis and amplification were used to evaluate TNF-α, IL-6, and GAPDH.

3.4.1. cDNA synthesis

The Max M-MLV Reverse Transcriptase First Strand for the PCR kit was used to reverse transcribe 5 μg of total RNA as per the manufacturer’s protocol.

3.4.2. PCR Technique

PCR was employed to directly amplify the first strand of cDNA synthesized in the previous method. A tenth of the original quantity (2 μl) was used. The primers used to amplify DNA from TNF-α and IL-6 are shown in Table 1. GAPDH, a housekeeping gene, was used as an internal standard for each sample. A 50 μl solution was prepared, including 10x PCR buffer with (NH4)2SO4, MgCl2 2 mM, 0.2 μM dNTP mix, autoclaved water, and 0.2 μM forward and reverse primers per reaction. The process of MJ research PTC200 in a thermal cycler was used to perform PCR amplification. The PCR reaction for TNF-α and GAPDH was carried out with an initial denaturation of 2 min at 95°c followed by 34 cycles of denaturation at 95°c for 20 s, annealing at 60°c for 30 seconds, and elongation at 72°c for 40 second. The final extension was carried out at 72°c for 10 min. For IL-6, the PCR conditions consisted of an initial denaturation at 94°c for 1 minute followed by 34 cycles of denaturation at 94°c for 30

s, annealing at 60°c for 30 s, and elongation at 72°c for 90 s. At 72°C for 10 minutes, the last expansion was completed. Ethidium bromide staining was used to evaluate and identify the amplification products after electrophoresis in 1.5% agarose gel. A densitometry study was performed to evaluate the ratio of the quantities of IL-6 and TNF-α mRNA in comparison with GAPDH mRNA, and the molecular weight of the amplified cDNA was measured by comparing it with a standard molecular weight marker of 2 kb ladder.

3.4.3.TNF-α & IL-6 mRNA expression in agarose gel electrophoresis & PCR techniques:

A 1.5% agarose gel electrophoresis in 1x Tris–borate solution was used to quantify the PCR product. The identified bands were stained with ethidium bromide. The final assessment compared TNF-α and IL-6 to GAPDH expression in each sample and PCR agarose gel visual band (Image Quant LAS 500, GE Healthcare Life Sciences) were densitometric analysis using Image quant LAS500 software.

Table 1. The forward and reverse primer sequences were used to determine TNF-α, IL-6, and GAPDH mRNA expression in liver damage following paracetamol toxicity. All primers were designed from the National Center for Biotechnology Information (nih.gov) https://www.ncbi.nlm.nih.gov/ and IL-6 (GenBank accession no. NM_012589.2), TNF-α GenBank accession no. NM_012675.3) and GAPDH (GenBank accession no. NM_017008.4). National Center for Biotechnology Information.Ncbi.nlm.nih.gov. from https://www.ncbi.nlm.nih.gov/.

Primer	Forward (F) & Reverse primer (R)
IL-6	F 5` CACTTCACAAGTCGGAGGCT 3` R 5` AGCACACTAGGTTTGCCGAG 3`
TNF-α	F 5`GGCTTTCGGAACTCACTGGA3` R 5`CCCGTAGGGCGATTACAGTC 3`
GAPDH	F 5`GCATCTTCTTGTGCAGTGCC3` R 5`GATGGTGATGGGTTTCCCGT3`

Post-treatment of animal groups, endpoint agarose gel electrophoresis PCR evaluated the mRNA expression of TNF-α and IL-6. Total RNA was isolated, and PCR was performed. On a 1.5 % agarose gel, the PCR products were separated and stained with ethidium bromide. Imagequant software densitometric analysis was employed to quantify the mRNA expression of TNF-α, IL-6, TNF-α/GAPDH, and IL-6/GAPDH values were expressed as mean ± SD in triplicate investigations using Imagequant LAS500 software densitometric analysis.

3.5 Histopathological examination

Hematoxylin and eosin (H and E) were used to evaluate the effect of EP on paracetamol-induced liver injury. The liver tissue was first isolated, followed by paraffin embedding fixation and then dehydration by alcohol. Samples of 3-4 μm thickness were sectioned, deparaffinized, and rehydrated. It was then finally stained with hematoxylin and eosin. The samples were evaluated and identified liver cellular damage using the EVOS®-XL imaging system [24].

3.6. Isolation of primary hepatocytes:

Seglen's two-step perfusion in situ improved the inverse perfusion technique for obtaining primary hepatocytes from healthy rats. Before receiving paracetamol treatment, the hepatocytes were chosen and shown in a culture plate with 24 wells (5×10^4 cells per well) for 6 hours. The culture media were then changed to growing culture media without serum for 24

hours. The culture was incubated at 37 °C in a humid environment with 5% CO2 [25]. The isolated primary hepatocytes were divided into two separate sets. The 1st set of isolated primary hepatocytes was used for MTT assay, and the 2nd set was used for AST, ALT, ALP, and total bilirubin.

3.6.1 Isolation of protein (protein fraction) from EP

The crude earthworm was purified using size exclusion chromatography (Sephadex G-75) and salting out using ammonium sulfate. The earthworm autolysate (earthworms were homogenized and autolyzed at 50°C for 4 hours with 0.2% sodium azide added as a bacteriostatic) was filtered, centrifuged (12,000 g), and cold conditions were used to precipitate ammonium sulfate from the supernatant. After dialysis, the precipitate was suspended in a 50 mM phosphate buffer with a pH of 6.0. After being pre-treated with acid (1N HCl) and alkali (1N NaOH) at 80°C for 10 minutes, renatured in distilled water, and preserved in 20% (v/v) ethyl alcohol, the filtration membrane (viva spin, Merck) of MWCO 50 kDa was used. The protein samples were dialyzed for 4 hours against a 50 mM phosphate buffer with a pH of 6.0. Further, dialysis samples were combined and gathered in a sterile container, and all samples were freeze-dried for invitro use. The Lowry technique was used to measure the amount of the protein fraction of MWCO 50 kDa. Protein fractions with MWCO 50 kDa were separated using SDS-PAGE [26].

3.6.2. Determination of cellular viability in MTT assay

A preliminary study on earthworm protein MWCO 50 kDa powder was conducted to assess viability in primary hepatocytes using earthworm protein MWCO 50 kDa powder. The earthworm protein MWCO 50 kDa powder was used for the MTT assay and was prepared in different concentrations of 5, 25, 50, and 100 mg/ml. With each earthworm protein MWCO 50 kDa dose, 19 mM/L of paracetamol, in 0.05% DMSO, was incubated except in the control group [25][27-28].

3.7. Hepatoprotective effect of protein fraction in in-vitro Experiment

The primary hepatocyte culture was randomly allotted to six groups. 1) Control group or Group (I), 2) paracetamol-induced disease control group or group (II) 3) Low concentration of earthworm protein MWCO 50 kDa (10 mg/mL) or Group (III), 4) Medium concentration of earthworm protein MWCO 50 kDa (50 mg/mL), or Group (IV), 5) High concentration of earthworm protein MWCO 50 kDa (100 mg/mL), or Group (V) 6) Standard silymarin group (SLY, 100 mg/mL) or Group (VI). The cells were pretreated with three different doses of the earthworm protein MWCO 50 kDa concentrations, while the silymarin group was treated with (100 mg/ml) of silymarin. The control group was treated with an equal volume of culture medium. After 12 h, the culture medium was replaced, and each group was treated with 19 mmol/L of paracetamol except the control group. The supernatant was collected after 10 h to evaluate AST, ALT, ALP and total bilirubin [25].

3.8. SDS-PAGE-based separation of MWCO 50 kDa protein fraction

The EP dialysate was subjected to gel filtration using Sephadex G-75, and the obtained filtrate was subjected to spin column for MWCO 50 kDa separation. The filtrate from Sephadex G-75 and MWCO 50 kDa fraction was collected and analyzed using mini gel SDS PAGE. Sephadex-G 75 beads were soaked in 50 mM phosphate buffer pH 6.0 for 48 hours while being slowly stirred to prevent air from being trapped in the matrix. The completely swollen beads were put onto a (30 ×2 cm) polypropylene column. Sephadex-G75 beads were packed under gravity with a repeated flow of phosphate buffer to prevent air entrapment and bed cracking. To accomplish compact packing, the column was packed for the dimension of 15 × 2 cm and kept for 24 hours at 10°C. The EP dialysate was placed into the Sephadex-G 75 column, and 1 ml/min of 50 mM phosphate buffer, pH 6.0, was used to elute the fraction [29]. The sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to assess the purity of earthworm protein's average molecular weight (SDS-PAGE). 10µl of pure filtrate

protein from sephdex-G75 and MWCO 50Kda fraction was placed into a 15% polyacrylamide gel with a standard range protein ladder. The protein bands on the gel were visible according to the Coomassie brilliant blue G-250 staining. Using a molecular mass marker kit, the protein's molecular weight was identified (6-200 kDa Mw range, Takara Japan) in both the spandex-G75 and MWCO 50 KDa fraction.

3.9. Statistical analysis:

All the data were analyzed using GraphPad Prism 9 (Graph Pad Prism, San Diego, CA, USA). The data were expressed as a mean ± SD. One-way analysis of variance (ANOVA) was used followed by Tukey's test. A value of ($P<0.05$) was considered statistically significant.

3.10. Result

3.10.1 Protein estimation:

Using the Lowry method, the total protein content in the EP and earthworm protein MWCO 50 fraction was estimated and was found to be 314.27±1.4 mg/dl and 125.42 ±0.6 mg /dl (Mean ± SD), respectively.

3.10.1. EP attenuated liver toxicity markers induced by paracetamol:

Fig. 1(a)-1(d) and 7(a)-7(d) represent AST, ALT, ALP, and total bilirubin levels, respectively in both in-vivo and in-vitro studies. Oral administration of 750 mg/kg b.w. of paracetamol weekly twice for 14 days resulted in a significant increase ($p<0.05$) in the activities of these enzymes as well as total bilirubin of paracetamol treated group rats when compared to the normal control group. The level of the tested enzymes and total bilirubin in the paracetamol-EP-treated group and paracetamol-silymarin-treated animals were significantly lowered ($p <0.05$). The statistical changes in the serum level of the biochemical parameters and enzymes of the liver in the paracetamol-silymarin treated group were more than the paracetamol-EP

treated group on the comparison. Similarly, all the AST, ALT, ALP, and total bilirubin were significantly ($p<0.05$) restored in the invitro experiment fig.7a-7d after earthworm protein MWCO 50 kDa fraction (group III-group V) treatment.

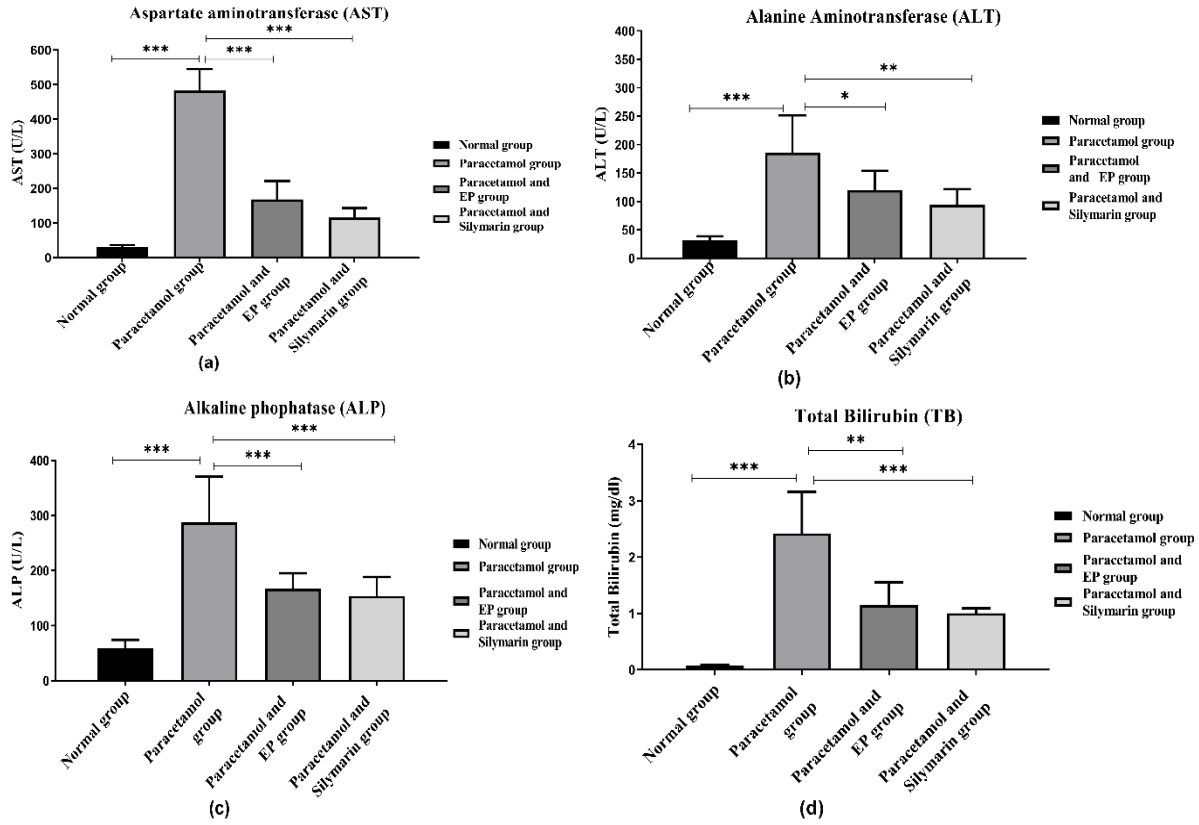


Figure 1: Effect of the EP (earthworm protein) on rat blood serum level (a) AST (U/L), (b) ALT (U/L), (c) ALP (U/L), and (d) Total bilirubin (mg/dl) concentrations were expressed respectively. Statistical comparison was made between the normal group Vs. Paracetamol-treated group the paracetamol-treated group Vs. Paracetamol and EP protein-treated group and the paracetamol and silymarin (reference drug)-treated group were found to be statically significant where *($p < 0.05$), **($p<0.01$) and ***($p<0.001$). Data are presented as mean ± SD (n=6) and analyzed by a one-way ANOVA test followed by Tukey's post hoc test.

3.10.2. EP attenuated oxidative stress markers induced by paracetamol:

In (Fig. 2a &2d) demonstrate the antioxidant effects of the EP on the rat liver tissue. The animals of the paracetamol-EP group showed significant ($p<0.05$) restoration of the levels of antioxidants, namely LPO (MDA), SOD, catalase (CAT), and GSH. The levels of SOD, catalase, and GSH were found to be significantly ($p<0.05$) elevated in the paracetamol-EP group and paracetamol-silymarin group as compared to the animals of the paracetamol-treated group. The liver tissue levels of LPO estimated as MDA in the paracetamol-EP group and the paracetamol-silymarin group were found to be significantly ($P<0.05$) decreased compared to the animals of paracetamol treated group. The restoration of these parameters was more pronounced in the paracetamol-silymarin group than in the paracetamol-EP group.

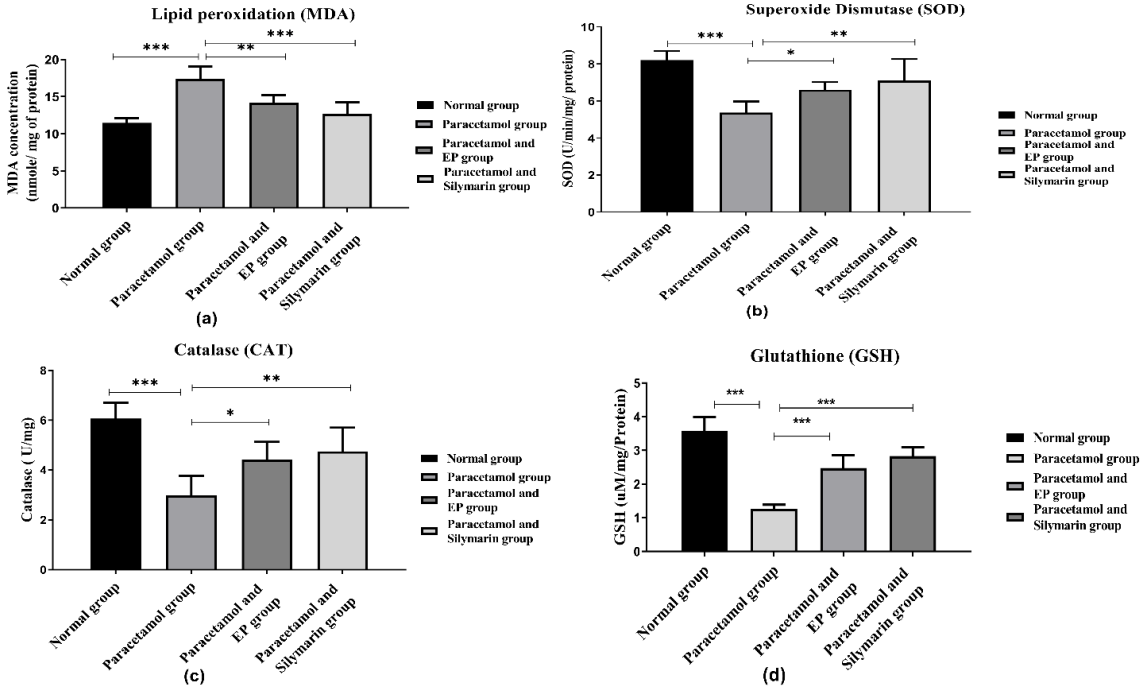


Figure 2. Effect of EP (earthworm protein) on liver tissue level of (a)MDA, (b) SOD, (c) CAT, (d) GSH. Statistical comparison was made between the normal group Vs. Paracetamol-treated group, paracetamol-treated group Vs. Paracetamol and EP protein-treated group, and paracetamol and silymarin (reference drug)-treated group were found to be statically significant where *($p < 0.05$), **($p<0.01$) and ***($p<0.001$).

3.10.3. EP attenuated paracetamol-induced histopathological changes:

The livers were stained with hematoxylin and eosin (H&E) to reveal typical hepatic architecture, which consisted of cords of hepatocytes with acidophilic cytoplasm and a vesicular nucleus positioned in the centre, separated from one another by blood sinusoids. Kupffer cells (KP) that are phagocytic and endothelial lined the blood sinusoids. The portal triad, which included a sizable portal venule and a bile ductile, appeared normal in the animals of the normal group (Fig.3). In the paracetamol-treated group, liver tissue sections stained with H&E revealed that the normal architecture of the hepatic parenchyma had been lost, as well as the central vein had widened and that the hepatocytes had deteriorated with pyknotic nuclei and vacuolated cytoplasm. The nuclei of apoptotic cells were distinctively stained darker. Additionally, binucleated hepatocytes and dilated expression sinusoids with many Kupffer cells (KP) were seen. Massive inflammatory (MI) cellular infiltration and bile duct (BD) proliferation and hyperplasia were both seen in the portal region. Hepatic H&E-stained sections from the paracetamol-EP and paracetamol-silymarin treated groups revealed a remarkable improvement, as most of the hepatocytes were practically normal with no cytoplasmic deterioration. Although the sinusoids used to separate the hepatocytes were essentially normal, binucleated hepatocytes were still visible in paracetamol-EP and paracetamol-silymarin groups (Fig 3a). In (Fig 3b) represents the necrosis, inflammation, and steatosis in terms of histopathological score. Necrosis, inflammation, and steatosis were significantly ($p<0.001$) elevated in the paracetamol-treated group. The three parameters were significantly reduced ($p<0.01$) in paracetamol-EP and paracetamol-silymarin-treated groups.

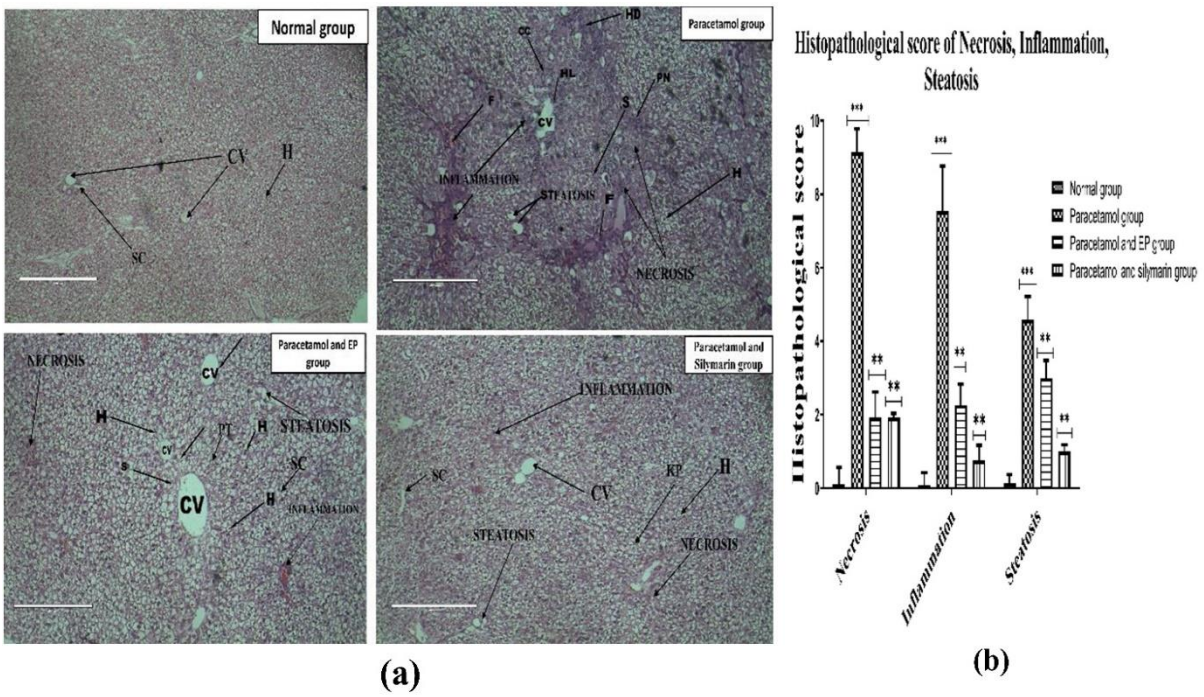


Figure 3. (a) Histopathological image of a Wistar rat liver (H&E, 10 X). The normal Group of the Wister rat liver sections was characterized by normal hepatic sinusoids (S), hepatic zonation, and central vein (CV). No fibrosis development was noted, and hepatocytes (H) were of appropriate shape. Paracetamol The group showed Steatosis (ST) marked by arrow signs that indicate fatty changes (F), cells with lost nucleus like the pyknotic nucleus (PN), numerous grouped apoptotic cells, Sinusoidal Space (S) expansion, stellate cells change (SC), Destruction of Hepatocytes(H), Zonation changed, more iron deposition, bile duct hyperplasia (HL), these changes were necrotic, binucleated hepatocytes and dilated, expression sinusoids with many kupffer cells (KP) were seen. Massive inflammatory cellular infiltration, bile duct (BD) proliferation, and hyperplasia were both seen in the portal region and degrading. Hepatocyte (H) disruption was evident in the paracetamol-treated group compared to the normal group. No fibrosis occurring sign developed, no structural and central vein (CV) change in shape in the paracetamol-EP treated group was found and showed restoration of the histoarchitecture. The paracetamol-silymarin-treated group showed more reduction in hepatic injury than the paracetamol-EP-treated group. (b) Represents

histopathological changes in terms of score, taking into account Necrosis, inflammation, and steatosis. Statistical comparison was made between the normal group Vs. Paracetamol-treated group, paracetamol-treated group Vs. Paracetamol and EP protein-treated group, and paracetamol and silymarin (reference drug)-treated group where **($p<0.01$) and ***($p<0.001$).

3.10.4. The EP attenuated elevation of TNF-α and IL-6 mRNA expressions induced by paracetamol:

End-point agarose gel PCR was used to evaluate the inflammatory gene expressions of TNF-α and IL-6 in acute liver-damaged rats. TNF-α and IL-6 mRNA expressions were significantly ($P<0.05$) greater in animals of the paracetamol group than in the normal group, as indicated in Fig 4: (a), (b), (c). The mRNA expression levels of TNF-α and IL-6 were significantly lowered ($P<0.05$) in the paracetamol-EP and paracetamol-silymarin groups compared to the paracetamol-treated group. However, it was observed that the reduction in the expressions of TNF-α and IL-6 in the paracetamol-silymarin group was slightly more than that of the paracetamol-EP group (Fig 4). All the values were triplicated in the Image quant LAS500 software in densitometric analysis.

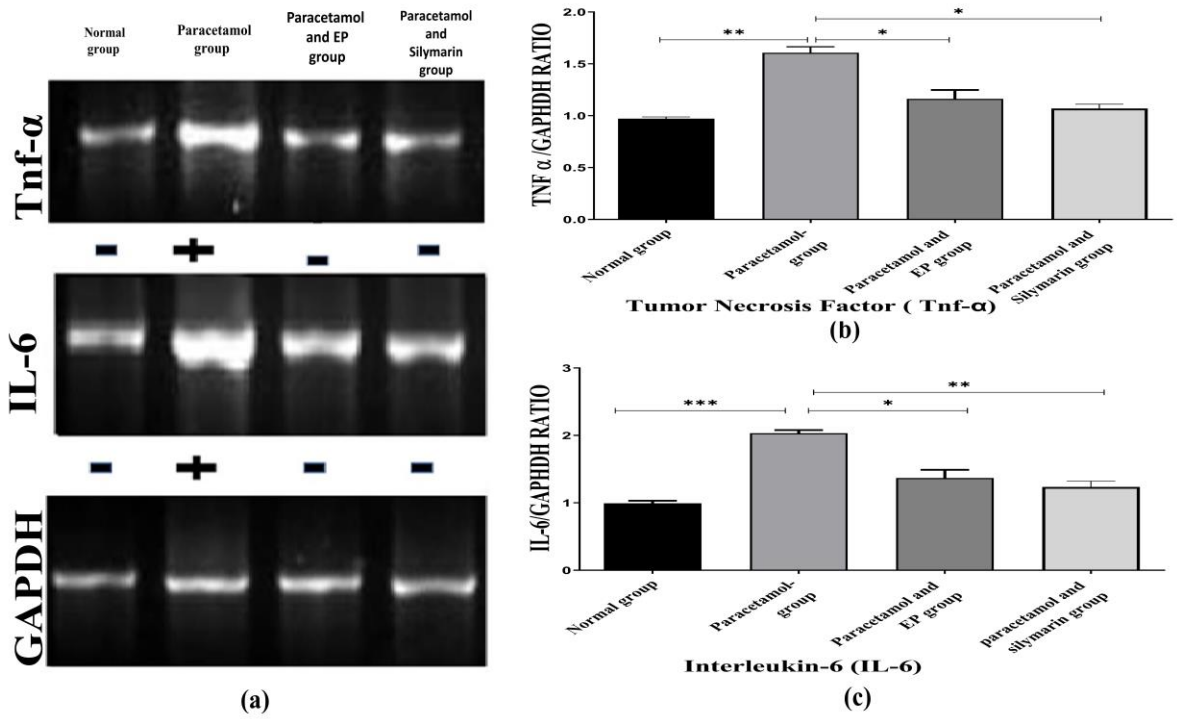


Figure 4. (a) Represents TNF-α, IL-6, and GAPDH gel doc Image (Image quaint LAS500 software densitometric analysis). The mRNA expressions of (b)TNF-α (c) IL-6 in all four groups are also presented. Statistical comparison of the mRNA expression of TNF-α and IL-6 was performed between the Normal group vs. paracetamol-treated group, paracetamol-treated group vs. paracetamol-EP treated group, paracetamol treated group vs paracetamol-EP treated group and paracetamol-silymarin treated group which significantly changed. The data represented as Mean of triplicate ±SD where *($p<0.05$) **($p<0.01$) and ***($p< 0.001$). Here, (a) (+) and (-) signs indicate the expression intensity bands of TNF-α, IL-6, and GAPDH.

3.10.5. Percentage of inhibition of antioxidants and oxidative stress markers by EP:

The percentage of inhibition in paracetamol-EP group and paracetamol-silymarin group for AST, ALT, ALP, Total Bilirubin, GSH, MDA, SOD, Catalase, (Fig. 5) respectively increase from 69% to 81%, 38% to 59%, 52% to 58%, 66% to 78%,52.26% to 67.48%, 44% to 66%, 60% to 84%, 63% to 77%, (Fig 5).

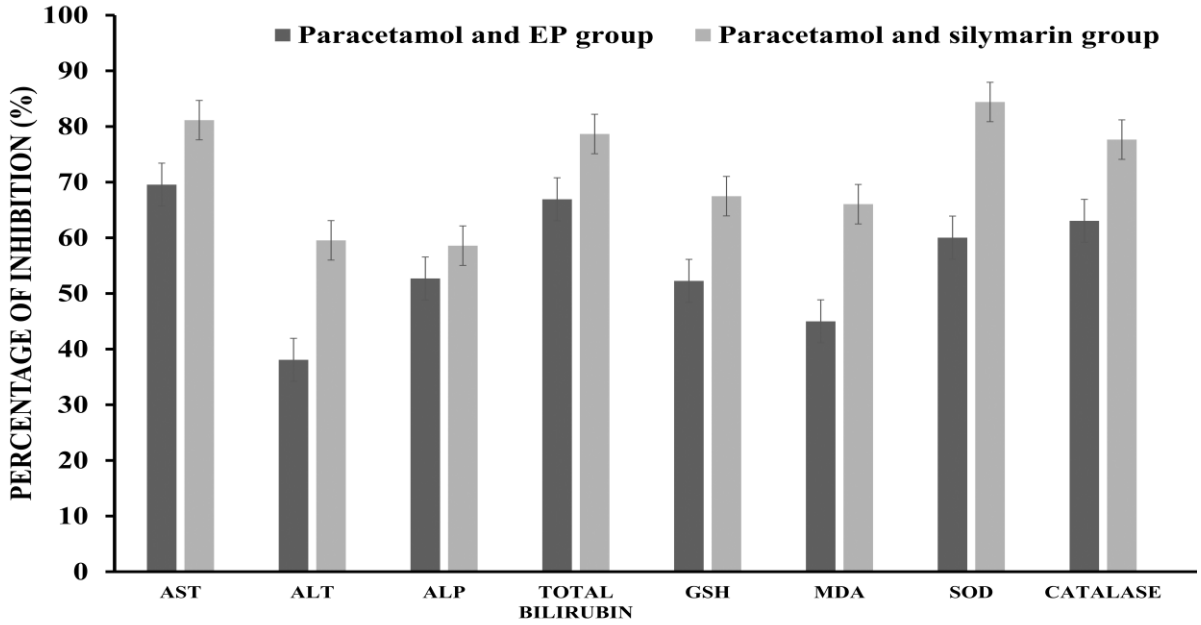


Figure 5: Represent the percentage of inhibition of all the estimated liver biochemical parameters and oxidative stress markers in both paracetamol-EP treated and paracetamol-silymarin treated group compared to the paracetamol-treated group, which is considered 100%.

3.10.6. Effect of earthworm MWCO 50 kDa protein on cell viability MTT assay:

After 24 hours of incubation, the effects of various doses (5, 25,50,100 mg/ml) of earthworm MWCO 50 kDa protein were assessed using the MTT assay (Fig.6). Primary hepatocyte culture media was incubated and treated with 19 mM paracetamol in 0.05% DMSO for 24 hours. The in-vitro viability assay indicates the % hepatocyte viability increases with increasing dose of earthworm MWCO 50 kDa.

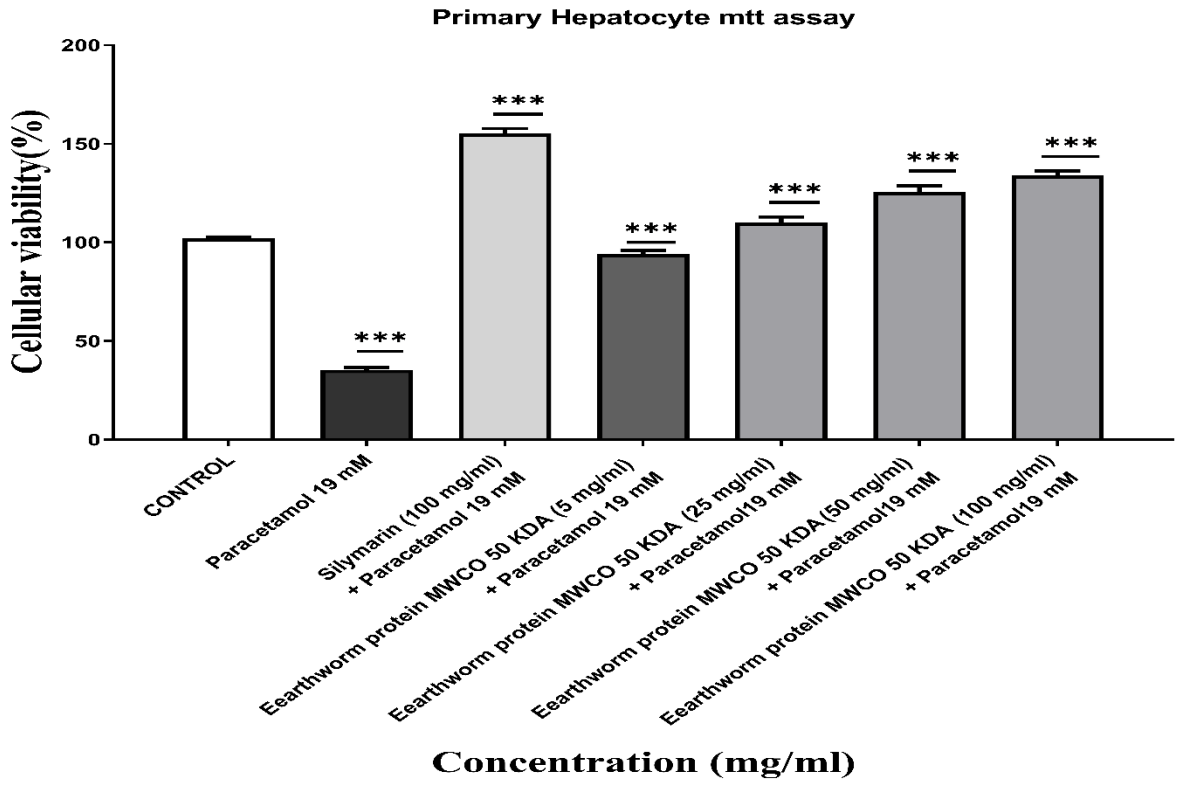


Figure 6: Cell viability of earthworm protein MWCO 50 kDa (dialysate) on rat primary hepatocyte. Rat primary hepatocytes were treated with Control (no treatment), paracetamol 19 mM (disease control), (5-100) *m*g/ml earthworm protein MWCO 50 kDa with paracetamol 19 mM and Silymarin (SLM) (100 mg/ml+19 mM) for 24 hours. % Cell viability was measured by MTT assay, described under the materials and methods section. The data are presented as the Mean of triplicate ±SD. Statistical comparison was made between the control group Vs. paracetamol 19mM group, paracetamol 19mM group Vs. silymarin (100 mg/ml) +paracetamol 19mM group, paracetamol 19mM group Vs. earthworm protein MWCO 50Kda (5mg/ml)+ paracetamol 19mM group, paracetamol 19mM group Vs. earthworm protein MWCO 50Kda (25mg/ml)+ paracetamol 19mM group, paracetamol 19mM group Vs. earthworm protein MWCO 50Kda (50mg/ml)+ paracetamol 19mM group and paracetamol 19mM group Vs. earthworm protein MWCO 50Kda (100mg/ml) + paracetamol 19mM group where ***($p<0.001$).

3.10.7. Earthworm MWCO 50 kDa protein attenuated liver toxicity markers induced by paracetamol on primary hepatocytes:

In Fig.7, the paracetamol-induced disease control group or Group (II), in comparison to the control group (I), showed a significant ($p<0.05$) increase of AST, ALT, ALP, and Total bilirubin levels in the supernatant. As compared to the paracetamol-induced disease control group (II), a significant ($p<0.05$) decrease in the silymarin Group (VI) was noted in the supernatant level for the mentioned evaluated parameters. Further, a significant ($p<0.05$) decrease in all the different concentrations (10,50 and 100 mg/ml) of earthworm protein MWCO 50 kDa group (group III- group V) was observed in all the biochemical parameters. Total Bilirubin in low-concentration earthworm protein MWCO 50Kda (10 mg/ml) or group III and medium-concentration earthworm protein MWCO 50kDa (50 mg /ml) or group IV was found to decrease but non-significantly.

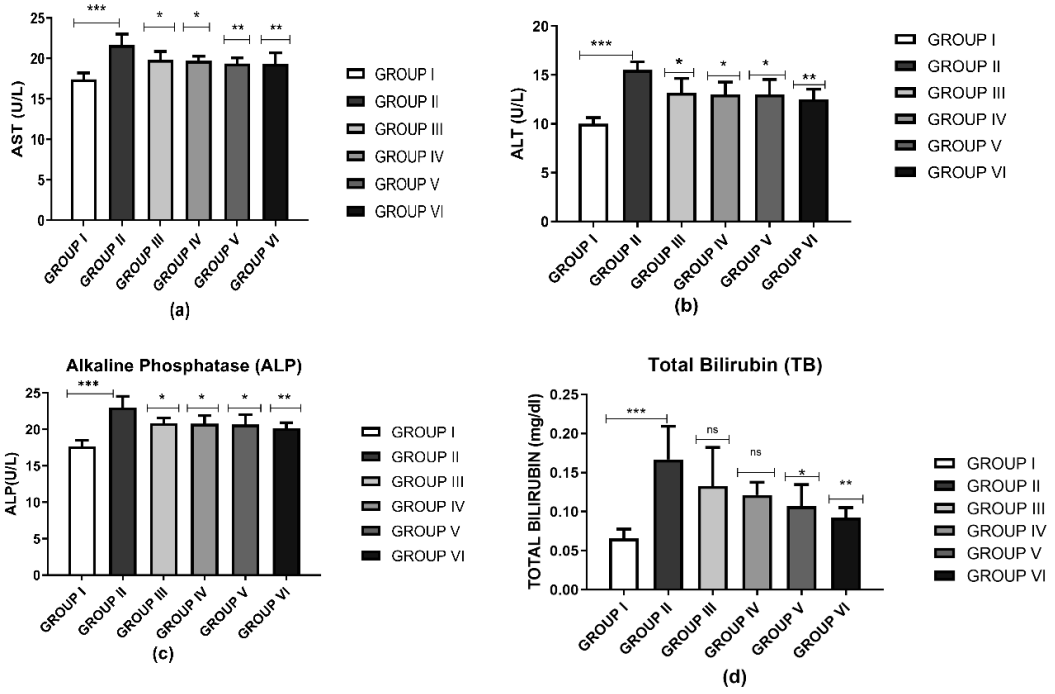


Figure 7: The changes of ALT, AST, ALP, and Total bilirubin activities in primary hepatocyte culture supernatant. The control group, or group I, was compared with the paracetamol-

induced disease control group or group (II). The Paracetamol-induced disease control group or group (II) was compared with the Low concentration of earthworm protein MWCO 50 kDa (10 mg/mL) or Group (III), Paracetamol-induced disease control group or group (II) vs. medium concentration of earthworm protein MWCO 50 kDa (50 mg/mL) or Group IV, Paracetamol induced disease control group or group (II) Vs. High concentration of earthworm protein MWCO 50 kDa (100 mg/mL) or Group V and Paracetamol induced disease control group or group (II) Vs. Standard silymarin group (SLY, 100 mg/mL) or Group (VI). Values were expressed as mean ± SD in each group where ns means non-significant, *($p<0.05$), **($p<0.01$), ***($p< 0.001$).

3.10.8. SDS PAGE protein identification:

We first performed protein purification from Sephadex G-75 by SDS-PAGE analysis. After that, we collected proteins from 50 kDa MWCO to below 50 kDa and analyzed them on SDS PAGE. These proteins below 50 KDA were tested in vitro with paracetamol toxicity of AST, ALT, ALP, and total bilirubin in primary hepatocytes at different concentrations.

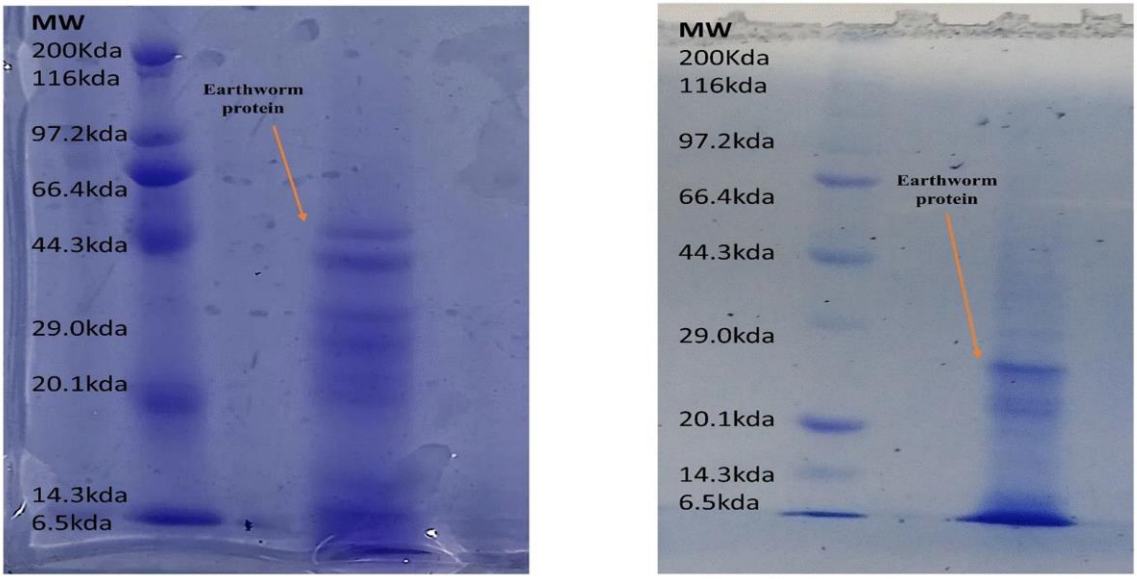


Figure 8: The molecular weight determination of purified earthworm protein. (Fig 8a) after gel filtration (Sephadex g-75) in SDS PAGE. (Fig 8b) showed below MWCO 50 kDa protein band showed in SDS-PAGE.

3.10. Discussion:

The use of Earthworms finds its root in both Chinese and Indian traditional medicine systems. The local (Dattapukur, West Bengal, India) use of this particular species *Pheretima posthuman* (also known as *Metaphire posthuman*), has been prevalent for a long time. From this perspective, the earthworm used in the Indian traditional medicine system has a co-relation with the Chinese traditional medicine system, though the hepatoprotective and regenerative activity of this particular species, i.e., *Pheretima Posthuma*, has not been reported yet. Our hypothesis regarding the hepatoprotective and regenerative ability of *Pheretima posthuma* (Indian earthworm) turned out to be promising, as was indicated through the attenuation of mRNA expressions of IL- 6 and TNF-α as well as restoration of biochemical parameters in paracetamol-induced liver injury through NAPQI generation. The NAPQI, produced due to paracetamol overdose, damages the cellular permeability of hepatic

cells by causing lipid peroxidation [30-33]. In turn, the hepatocytes liberate their content, leading to elevated blood levels of ALT and AST, as shown in our experiment (Fig 1a&1b). As seen in Figs 3a and 3b, EP treatment significantly ($p<0.05$) reduced serum ALT and AST, consequently guarding against hepatocytic necrosis. EP revealed considerable protection against the development of hepatic steatosis in (Fig 3a &3b), which is known to arise owing to paracetamol-induced impaired liver lipid metabolism. This finding may be related to EP's ability to protect hepatocytes from necrotic damage caused by paracetamol, which is due to the stabilization of the hepatic cell membrane, resulting in less leaching of cellular contents and finally expressed as lower levels of released transaminase enzymes in plasma. In the current discussion, it is vital to note that paracetamol affects hepatic lipid metabolism, resulting in higher blood triglycerides and irreversible inhibition of fatty acid -oxidation. Consequently, accumulating intracellular lipids leads to steatosis, a hallmark of liver disease [34]. After that, the progression from steatosis to non-alcoholic steatosis hepatitis is influenced by several factors, including lipid accumulation, oxidative stress, cytokines, and the production of proinflammatory mediators [35]. As a result, EP can be predicted to prevent the progression of paracetamol-induced steatosis (Fig 3a&3b) to non-alcoholic steatosis hepatitis. According to scientific evidence, paracetamol-induced toxicity causes increased heme breakdown and bile tract obstruction. As a result, the conjugation reaction is halted, and the injured hepatocytes produce unconjugated bilirubin, resulting in serum hyperbilirubinemia [7], [36]. EP displayed hepatoprotection against paracetamol hepatic insult in our investigation. As a result, it is plausible that, besides other hepatoprotective effects, EP might lessen the degree of heme breakdown [37] and consequent biliary tract obstruction, resulting in decreased serum hyperbilirubinemia. In addition, mitochondria have been shown to play a crucial role in the pathophysiology of liver injury and acute liver failure caused by paracetamol overdose. Hepatic glutathione depletion, mitochondrial protein

adducts, and initial oxidant stress result from an overdose of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which in turn activates the mitogen-activated protein (MAP) kinase cascade and subsequently causes c-jun N-terminal kinase (JNK) phosphorylation. When oxidative and nitrosative stress is amplified, mitochondrial membrane permeability transition pore opening and ATP synthesis shut down due to phospho-JNK translocation to the mitochondria. In addition, the release of endonucleases leads to DNA fragmentation due to the bursting of the outer membrane of the mitochondria. The combination of widespread mitochondrial dysfunction and nuclear DNA damage leads to necrotic cell death. [38-40]. In this context, it will be interesting to mention that increased necrosis (Fig. 3a and 3b) and GSH depletion (Fig 1d) have been observed in our study in the paracetamol-treated group. Furthermore, following hepatic injury, hepatic mitochondrial DNA (mt-DNA) replication is required for the essential regeneration of damaged liver tissue, where the enzyme 'Topoisomerases' plays a crucial role, amongst many other critical factors. Prior research suggests that NAPQI inhibits the enzyme topoisomerases, reducing the necessary replication of mt-DNA [41], [33]. In our work, the paracetamol-EP treatment group restored GSH levels (Fig 2) and improved hepatic necrosis (Fig 3a&3b), suggesting that EP may have a role in restoring hepatocytic mitochondrial activity via the mechanisms mentioned above of action (s). Apart from the explanations above, another major aspect is that paracetamol induces lipid peroxidation, resulting in the oxidation of unsaturated fatty acids in the hepatic cell membrane and, eventually, leakage of cellular content. An elevated level of malondialdehyde (MDA) is a sign of such hepatic cell membrane lipid peroxidation, and as previously stated, cellular content leakage is associated with elevated blood transaminase enzymes. As a result, in our findings, paracetamol increased MDA levels, but paracetamol-EP reduced MDA levels significantly, indicating a protective function to prevent cell membrane rupture. SOD and CAT are two endogenous antioxidant systems that are

thought to be responsible for organisms' defensive response to oxidative stress. A similar trend was found in our experiment with SOD and CAT owing to the generation of NAPQI and eventually ROS, the decline of which due to paracetamol injury in the paracetamol treated group was reported to be significantly ($p<0.05$) preserved by EP treatment in the paracetamol-EP group In an attempt to address broader occurrences of other documented cellular activities, the toxic metabolite NAPQI generated by paracetamol stimulates Kupffer cells, producing the release of the inflammatory cytokine TNF-α, which also significantly contributes to cell death. It is critical to note that apoptosis and necrosis generally coexist in liver pathology, and the cell death balance can be affected by chemical or pharmacological assault [39], such as paracetamol. According to previous research, paracetamol increases hepatic TNF-α and NF-κB DNA binding, which is associated with increased cyclin-D1 protein synthesis and liver regeneration in mice [40]. Together with IL-6, a multifunctional cytokine with pathogenic and pro-inflammatory functions in autoimmune disease animal models [33], [42-43]. Further reports claim that the functioning of the IL-6/STAT-3 signalling pathway is responsible for the hepatic regeneration process following paracetamol-induced liver injury. An overdose of paracetamol in mice was linked with higher levels of IL-6 in both the liver and the serum [44-46]. According to our findings, the higher expression of TNF-α and IL-6 in the paracetamol-treated group might be an indication of inflammation, stress, or the activation of the regeneration process, whereas the paracetamol-EP treated group showed a significant ($p<0.05$) reduction in TNF-α and IL-6 (Fig 4). The histopathological score of decreased inflammation (Fig 3a and 3b) validated the paracetamol-EP group's significant ($p<0.05$) reduction in inflammatory markers. According to Zhu et al., the liver tends to maintain homeostasis about the size [47-48], where initially elevated levels of IL-6 can swiftly upregulate the expression of SOCS3. Following that, SOCS3 can limit subsequent IL-6 signalling pathways (by inhibiting STAT3 phosphorylation) [49]. The reduction in IL-6

observed in the paracetamol-EP group may indicate the completion of the regeneration process as well as a reduction in inflammation, which might have eventually led to the stated homeostasis of liver size, but it cannot be denied that IL-6 and TNF- are not the only players in liver regeneration. The MTT assay performed with primary hepatocytes involving different doses (5,25,50 and 100 mg/ml) of earthworm protein MWCO 50Kda significantly increased ($p<0.05$) viability in a dose-dependent manner (Fig.6), suggesting an increase in primary hepatocyte proliferation. In a similar set of primary hepatocyte cell cultures against paracetamol insult, liver biochemical parameters (AST, ALT, ALP, and Total Bilirubin) were found to be restored (Fig 5). EP may protect hepatocytes from paracetamol injury, prevent mitochondrial dysfunction and boost hepatic regenerative ability. It will be justified to mention that to create a real-time situation or to relate gastric biomembrane transit of our EP product; we have used the EP dialysate (using 14kDa Membrane). After that, following different fractionation using a spin column, the MWCO 50 kDa and lower molecular weight fraction(s) [29] were found to be more potent. Considering this approach with *Pheretima Posthuma*, we feel it is much more important to correlate observed in vivo and in vitro hepatoprotection of EP and its scientific justifications with real-time usage.

Conclusion

Our report with EP against paracetamol-induced hepatocellular damage confirms previous claims [14] involving the reduction of oxidative stress and inflammation. However, the genus and species of earthworm mentioned earlier was different to those of our study. Moreover, EP (*Pheretima posthuma*) activities involving cytokine expressions e.g. TNF-α and IL-6 using lower molecular weight protein fraction from the dialysate i.e. MWCO 50 kDa and below protein fractions is of scientific importance with correlation to real time use. This study will help to carry on further investigation to find our more feasible or better cocktail of EP protein fraction / fractions to be used as hepatoprotective product.

Acknowledgments

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Ethical Statement

The Institutional Ethical Committee (established under the guidelines Committee for the Purpose of Control and Supervision of Experiments on Animals, Reg. No. AEC/PHARM/1702/3/2017) strictly supervised the care and usage of the animals.

Funding Statement

We are thankful to the the UGC (Rajiv Gandhi National Fellowship, F117.1/201415/RGNF2014-15SCWES78683 /(SAIII/Website), India for funding this project. We are also thankful to the Bioequivalence Study Centre, Department of Pharmaceutical Technology; Jadavpur University for providing us required facilities to carry out the work. Authors are also thankful to Central Council for Research in Unani Medicine (Ministry of AYUSH) UGC-DSA, DST, UGC-UPE, RUSA 2.0, India.

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CHAPTER IV

Natural hepatoprotectives earthworm extract protein & goat milk *in-vitro* model rat primary hepatocytes exposed to carbon tetra chloride revealed toxicity and oxidative stress

Abstract

The assessment of bioactive ingredients with potential hepatoprotective properties focuses primarily on in-vitro bioassays. This study aimed to assess the effectiveness of pre-treating primary hepatocyte cells with hepatoprotective earthworm extract to prevent damage from carbon tetrachloride (CCl4). Cell viability, aspartate aminotransferase (AST), alanine aminotransferase (ALT),lipid peroxidation or malondialdehyde (MDA) levels and tumor necrosis factor (TNF-α) activities, as well as hepatoprotective activity of Earthworm extract protein powder (EEP) different concentration were 25, 50, and 100 µg/ml, Goat milk powder (GP) and Silymarin (SLM 100 µg/ml), and CCl4 were assessed in-vitro assay on the laboratory model rat primary hepatocyte culture. The selection of the CCl4 minimum lethal dosage might occasionally make examining liver damage more time consuming. Therefore, we examine CCl4 effectiveness at low to high doses in primary rat hepatocytes. Using primary hepatocyte as a toxicity model, we explored the cellular toxicity and oxidative stress related to CCl4 exposure. Our findings imply that the CCl4-induced cytotoxicity is fundamentally influenced by oxidative stress. The MTT assay results exhibited that CCl4 lower to higher doses decreased the viability of primary hepatocytes within six hours of treatment and hepatoprotective activity of EEP and EEP+GP was identified. The lipid peroxidation assay revealed that MDA levels were inhibited by EEP and GP treatment in primary hepatocytes. Our results also showed EEP and GP treatment significantly inhibited AST, ALT, inflammatory marker TNF-α, and MDA level increase due to inducing CCl4 in in-vitro rat primary hepatocytes. According to the studies, EEP and GP function as an efficient hepatoprotective agents and prevent CCl4 treatment-induced primary hepatocyte toxicity.

KEYWORDS: Hepatoprotective; Primary hepatocyte; Earthworm extract protein; Goatmilk; Silymarin

4. Introduction:

The liver is essential for maintaining detoxification and the metabolism of xenobiotics. Yet, throughout these activities, the liver is exposed to harmful chemicals that can cause liver damage or injury. Protein-based hepatoprotectives have benefits over medicinal plant bioactive substances. With high bioavailability, the body quickly absorbs amino acids from proteins. Due to inadequate absorption or rapid degradation, medicinal plant bioactive components may have decreased bioavailability. Focused activity Proteins may be chosen to target liver cells and influence hepatoprotective processes [1]. Their targeted activity improves liver protection and regeneration. Medicinal plant bioactive chemicals frequently influence several organs or systems, limiting their liver protective specificity. Tolerance and safety as part of regular physiological processes, the body tolerates proteins from natural sources like food or endogenous substances. They are safer than synthetic medications. Bioactive substances from therapeutic plants may cause unexpected side effects, medication interactions, and allergic responses [2]. Milk thistle, or Silybum marianum, is the source of the natural chemical silymarin [3]. Inhibiting the free radicals created during the metabolism of harmful chemicals including ethanol, acetaminophen, and carbon tetrachloride is what gives silymarin its hepatoprotective and antioxidant effect and makes it a standard candidate drug. It is well established that free radical production results in membrane damage and lipoperoxidation. Even the hepatoprotective flavonoid silymarin has drawbacks in managing chronic liver disorders, such as cirrhosis [4]. Hence, there is a need for novel therapeutic approaches that provide hepatoprotection and promote hepatocyte regeneration to complement or replace the existing treatment. According to our literature review [5] Milk-derived whey protein includes hepatoprotective bioactive peptides. Antioxidant, anti-inflammatory, and immune-modulating peptides protect the liver. Whey protein may help NAFLD and alcoholic liver disease. New drug mainly monoclonal antibody already treatment for specific liver disease. Albumin injection, a key plasma protein, treats liver problems—antioxidant, transporter, and oncotic pressure maintainer [6]. Albumin helps prevent hepatic encephalopathy in liver disease in some recent studies [1]. Hepatocyte Growth

Factor (HGF) is a protein that is naturally produced in the body and plays a crucial role in liver regeneration and repair. Ursodeoxycholic acid (UDCA), also known as ursodiol, is a hepatoprotective compound commonly used in the treatment of various liver disorders. It is a bile acid naturally found in small quantities in human bile. In spite of the fact that no medication has yet shown complete hepatoprotective or regenerative capacity [7]. As a result, liver pathological conditions have become one of the most prevalent health issues affecting humans [8]. According to the World Health Organization, more than 10 percent of the global population has a liver disease [9]. Hepatotoxicity is the consequence of accumulation of hazardous substances or toxic chemicals in the body that surpasses the hepatocytes' ability for detoxification and regeneration [10]. Drug abuse is the most common cause of hepatotoxicity [11,12]. The earthworm extract has been shown to have various positive pharmacological effects, including fibrinolytic and anticoagulative action, anti-inflammatory activity, anti-oxidative activity, peripheral nerve regeneration, bone regeneration, and wound healing, according to growing research [13]. Earthworms have several benefits, including advantageous pharmacological actions, abundant resources, and a low cost. However, there has been little comprehensive research on the pharmacological effects of earthworm active compounds [14]. The earthworm extract has several beneficial pharmacological properties, including fibrinolytic and anticoagulative action, anti-inflammatory activity, antioxidant activity, peripheral nerve regeneration, bone regeneration, and wound healing [15]. Unfortunately, very little study has been conducted on the pharmacological effects of earthworm-active ingredients. While their pharmacological actions have previously been shown to be anti-inflammatory and antioxidative, their potential for cellular growth mechanisms is immense. These data may suggest that the EEP protects the liver. In recently marketed protein powders to improve their taste dairy products are used. Goat’s milk is reported to have higher digestibility and lower allergenic properties compared to cow’s milk. [16-17]. In addition, these benefits may be further enhanced by using goat’s milk as a vehicle for delivering EEP protein. Carbon tetrachloride is acutely hazardous and carcinogenic in both in vitro and in vivo models. Free radicals are thought to develop as a result of cytochrome P450-mediated processes in the in vitro model CCl_4 [18]. Previous research

shown that the livers of rats fed CCl_4 revealed at 4 hours, some hepatocytes had completely lost their glycogen and had begun to accumulate lipid, while others had severely dilated rough endoplasmic reticulum (RER). alterations in pericentral hepatocytes' morphology at 6 hours, including glycogen depletion, an increase in RER, and RER dilation were seen [19]. Liver toxicity is the primary goal of acute toxicity studies in rats given oral exposure; renal damage also develops, although increasing dosages are required, in fact, acute toxicity symptoms have also been seen in the lungs and brain [20]. The liver and the central nervous system, however, appear to be the principal targets of short-term exposures in animals exposed by inhalation; hepatotoxicity and, to a smaller extent, nephrotoxicity are the predominant acute effects. Furthermore, comparative investigations on the effects of oral vs air exposure on liver damage in animals demonstrate that oral exposure has worse consequences [21]. CCl_4 absorption from the gastrointestinal tract, it occurs quickly absorbed and is badly impacted in nutrition [22]. After ingestion, inhalation, or dermal absorption, CCl_4 travels throughout the body, with the most considerable amount seen in the liver, brain, kidney, muscle, fat, and skin. There is a limited human sign on the carcinogenic consequences of CCl_4. Those people who worked in the chemical industry found inhalation experience to CCl_4 has been found in several types of liver disease [23]. In addition, a study has already done oral, inhalation, and parenteral introduction to CCl_4 has been found to develop hepatocellular carcinomas in animals [24]. In addition to medicines, genetics, age, lifestyle, and environmental variables contribute to hepatotoxicity development [25,26,27,28]. Acute liver injury (ALI) is characterized by the abrupt loss of liver function [10]. ALI is caused by a viral infection, hazardous drug consumption, and other stimulants [29]. Nonetheless, treatment options for ALI remain quite inadequate. Carbon tetrachloride (CCl_4) is a recognized hepatotoxin that induces ALI and promotes lobular necrosis and fat accumulation in experimental animals and this is thought to be due in large part to NF-κB activation in the liver [30] Because of its similar pathophysiology to liver disorders, CCl_4-induced ALI is often utilized to examine the course of liver diseases [31]. While there is a growing demand for medications to protect the liver, safety problems remain the primary worry with present medications. Thus, the search for efficient and dependable therapeutic

foods for hepatoprotection is quite promising. Although not specific for DILI (Drug induced liver injury), the blood levels of alanine aminotransferases (ALT), aspartate aminotransferases (AST), alkaline phosphatase (ALP), and total bilirubin (TB) continue to be the gold standard for identifying and categorizing liver injury [32]. Traditional medicine has a long history of treating illnesses with earthworms. For example, earthworms were used by people from Burma to cure fever symptoms, in Laos to treat smallpox, and by the ancient Chinese to treat blood clots, hemiplegia, and convulsions brought on by fever [14]. Studies in recent medicine have demonstrated the positive benefits of earthworms in both in vitro and in vivo models, with a more excellent knowledge of the mechanism supporting the capacity of earthworms to cure disease [33]. Earthworm extract (EE) was revealed by Fu et al. to have the ability to stimulate osteoblast activation and cell proliferation in culture media. Earthworm extract protein (EEP) has medicinal potential, utilizing interest from numerous sectors. EEP is the protein fraction of earthworm extract (EE), which is a complex combination of bioactive components, including proteins. Traditional Chinese medicine (TCM) and other traditional treatment systems utilize EE for its benefits to health. It can function as anti-inflammatory, immunomodulatory, wound-healing, and antioxidant. EEP research now includes therapeutic uses. In vitro cell culture research has examined how EE affects cellular processes and mechanisms. These studies examine EEP's effects on cell viability, proliferation, apoptosis, gene expression, and signaling pathways. EE physiological effects have also been studied in animals. These experiments assist researchers comprehend EE advantages and processes in living species. In such studies, EEP is administered to animals, and various parameters such as biochemical, haematological, histological, and behavioural changes are evaluated. EE is occasionally mixed with other compounds to boost its medicinal efficacy. EEP is often used with glycosaminoglycans and chondroitin sulfate. These combinations enhance joint function and cartilage health synergistically; hence they are used in joint health supplements [34]. Other combinations include herbal extracts or bioactive compounds that complement the therapeutic properties of EEP. EE with antioxidant-rich plant extracts may boost antioxidant activity. To promote digestion and gut health, EE has been tested with enzymes or probiotics. Overall, the development of EE

research involves studying its effects through in vitro and in vivo tests, the main objective to understand its mechanisms of action and potential therapeutic applications [35]. Chang et al. studies that EE enhanced migration in Schwann cells by increasing the production of proteolytic enzymes that break down the matrix [14,36]. The pulmonary fibrosis and inflammation brought on by silica inhalation in mice may be significantly reduced by intraperitoneal injection of EE [37]. Oxidative stress is the principal cause of liver damage [38]. The production of oxidative stress, mitochondrial dysfunction, and endoplasmic reticulum stress by metabolic activation of chemically active intermediate metabolites that covalently bind macromolecules may result in hepatocyte injury [39]. Current research has shown that the majority of human diseases are associated with oxidative stress, which enhances the importance of finding antioxidants as part of the treatment [40]. While their pharmacological actions have previously been shown to be anti-inflammatory and antioxidative, their potential for cellular growth mechanisms is immense. These data may suggest that the EEP protects the liver. Dairy ingredients are used to enhance the flavour of newly introduced protein powders. Compared to cow's milk, goat's milk is more easily digestible and less allergic. In addition, it has a greater concentration of short-chain fatty acids in milk fat, a greater concentration of zinc, iron, and magnesium, and antibacterial properties [41]. Orally or intraperitoneally given CCl_4 has a rat LD_{50} of 1-2 millilitres per kilogram (ml/kg). Mouse LD_{50} are 1.5-3 ml/kg of body weight through the same pathways. Several studies have reported hepatotoxicity in an in-vivo system of (0.5-2 ml/kg) body weight. The specific toxic dose of CCl_4 in vitro can vary depending on the cell or tissue type, the experimental conditions, and the endpoints being measured. Different concentrations of CCl_4 are used in various in vitro studies depending on the research objectives [42]. In an animal model, although the precise toxicity starting level in CCl_4 dosage in vitro has not been determined in several research. As a result, our research one of the aims to establish the lowest CCl_4 dosage that inhibits cellular cell viability in primary rat hepatocytes in vitro. Goat milk is a popular alternative to cow's Milk for those with cow milk protein allergies or lactose intolerance. Proteins, vitamins, minerals, and bioactive substances provide therapeutic properties [43]. Studies suggest goat milk has anti-inflammatory, immunomodulatory,

antibacterial, antioxidant, and gastrointestinal health effects. In Characterization and classification of goat milk and its components are used in functional meals, nutraceuticals, and pharmaceuticals because of these qualities. Caseins, whey proteins, immunoglobulins, lactoferrin, and bioactive peptides are goat milk proteins that have been widely investigated. These proteins have particular bioactivities that may make goat milk healthy [44]. In vitro and in vivo research is being conducted on goat milk and its proteins to discover their mechanisms and therapeutic applications. Combining goat milk with other ingredients may increase its therapeutic efficacy. Probiotic goat milk improves intestinal health, digestion, and nutrient absorption. It's synergistic effects when coupled with herbal extracts or bioactive compounds such as polyphenols or omega-3 fatty acids [45]. Goat's milk has been reported to exhibit hepatoprotective activity in both CCL_4 [41], and anti-tubercular induced [46] Till date, there is no evidence of the hepatoprotective effects of EEP and GP combination. However, individually there are some reports indicating their hepatoprotective effect, though our species *Pheretima Posthuma* (*Metaphire Posthuma)* has not been explored yet. In our investigation, we created a model of EEP and EEP+GP induced primary hepatocytes in primary hepatocyte cultured liver cells, observed in the protective effects, and investigated the underlying processes after 6 hours in 40 mM CCl_4-induced damaged hepatocytes. One of the objectives was also to determine the LD_{50} value using toxicity data derived from this experiment.

4.1. Material

Carbon tetrachloride (Sigma chemicals) (Cat no,289116, Cell culture media Dulbecco Modified Eagle Medium (DMEM) (GIBCO) (cat. No: 11710035), HEPES buffer product no; H0887, sodium pyruvate product no; 23569 and sodium bicarbonate were purchased from Thermo Fisher science, product no; S8761, Fetal bovine serum (FBS) Thermo fisher, product no; F7524, antibiotics (penicillin G and streptomycin) Thermofisher product no; P4333, phosphate-buffered saline (PBS), SRL, product no; 78529. and MTT were obtained from Sigma Chemical, product no. M2003-1G. Amicon Ultra-15 Centrifugal Filter (cat no R1AB93508), TNF alpha Rat ELISA Kit (Cat no. BMS622). Silymarin was obtained sigma-

aldrich merck (cat no. S0292), Trichloroacetic acid (Sisco chemical, India), Thiobarbituric acid Thermo Fisher Scientific, India (cat. no.171740050), Hydrogen peroxide (cat. no.108600), phosphate buffer SRL India (cat. no.78529), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) (Product no.MAK052), we Penicillin-Streptomycin sigma-aldrich. Fetal Bovine Serum Thermofisher scientific (Cat.no.26140087). Spectra Max M5 Series Multi-Mode, Microplate Readers, Respons 910 autoanalyzer.

4.2. Extraction and purification of earthworm extract protein (EEP)

An earthworm breeding farm provided Indian earthworms (*Metaphire Posthuma*), and the Earthworm body parts were separated and purified as previously mentioned. Earthworms that had reached sexual maturity were briefly washed with tap water to get rid of the attached muck, and then they were homogenized using a tissue homogenizer. A tissue grinder was used to grind the homogenized material, which was then sonicated and centrifuged at 10,000 rpm for 10 min at 4 °C. The lower fraction remaining at the bottom of the centrifuge tube was ultra-centrifuged with 25 KDa ultra-filter (Millipore) at 4 °C after the supernatant had been ultra-filtered with 50 KDa ultra-filter (Millipore) with 5000 g for 20 min. Using Sephadex G-75, the ultrafiltered molecular cut-off (MWCO) extract was purified[60]. Phosphate-buffered saline (PBS, 0.1 M, pH 7.8) was used to elute the desired protein sample, then kept at 20 °C. The sample was centrifuged for 30 minutes at 1200 g in 4 °C. The pellets were dissolved in cold acetone to extract the remaining purified protein. The protein was lyophilized, then powder form protein was kept at 20 °C until used. The marketed lyophilized goat milk (GP) was used. EEP and EEP+GP, both lyophilized powders, were used with DMEMA in DMSO (0.05%).

4.2.1. Primary hepatocyte culture

In vitro assay to identify hepatoprotective effects. The hepatoprotective effects of EEP, Goat milk (GP), and Silymarin (SLM) on primary hepatocyte cells were measured as follows.

Normal control cells were incubated with DMEMA in DMSO (0.05% v/v) for 12 h. For toxic treatment, cells were incubated with DMEMA in DMSO (0.05% v/v) for 12 h and then treated with 40 mM CCl_4 for six h. For EEP and EEP+GP treatment, cells were incubated with DMEMA with EEP at 25, 50, and 100 µg/mL for 12 h and then treated with 40 mM CCl4 for six h. For SLM treatment, cells were incubated with DMEMA with SLM 100 µg/mL for 12 h and then treated with 40 mM CCl4 for six h. On the other hand, we also investigated the hepatoprotective effects of EEP combined with goat milk in this study. Perfusions of hepatocytes were performed in the Wistar albino male rats weighing 130 to 150 g. The method used was two-stage perfusion. At 37°C maintained solvent, the liver was firstly perfused with Ca2+ free 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) buffer (pH 7. 65) via the portal vein for 25 minutes. The solution was made up of the following ingredients: 160.8 mM NaCl, 3.15 mM KCl, 0.7 mM NaHPO412H2O, 2.05 mM EDTA, and 0.33 mM HEPES. The same solution was used for the second infusion stage with 0.025 % collagenase and 0.075 mM CaCl2 for 15 minutes. The liver was partially crushed in the HEPES buffer at the end of this two-step perfusion. Hepatocytes were purified by Percoll centrifugation and suspended in Dulbecco Modified Eagle Medium (DMEM) (GIBCO) at a concentration of 3×10^4 cells/ml. Hepatocyte viability was 95% after a trypan blue test. Before moving it to Petri dishes previously covered with collagen, DMEM in 10% (v/v) fatal bovine albumin (FBA) and antibiotics (100 units/ml penicillin) were used to create a new environment suspension. The culture was incubated for two hours at 37°C with humidity CO2/air. Hank's Balanced Salt Solution (HBSS) was then used to replace the culture medium. Finally, the primary hepatocyte culture was employed to experiment [61-62]. Each assay was done in triplicate.

4.2.2. Protective effect of EEP and EEP with GP MTT assay in primary hepatocyte

The cell viability was determined using an MTT assay. This colorimetric test uses mitochondrial succinate dehydrogenase, only found in living cells, to transform MTT into a

purple formazan color. The primary hepatocyte cultures were treated with EEP and EEP+GP at different concentrations. After the media was taken away, the cells were treated with MTT (0.5 mg/mL) for 4 hours, and then the formazan crystals were dissolved in 200 μL of DMSO each well. At 570 nm, the absorbance was measured using a Spectramax M5 96 microplate reader. Viability was determined as the percentage difference between the absorbance of treated cells and the absorbance of the control cells that were not treated. [61] .

4.2.3. Assay of Lipid Peroxidation (LPO) or (MDA)

As evaluated by lipid peroxidation levels in rat primary hepatocytes, CCl_4 treatment significantly increased lipid peroxidation. Ohkawa et al. established a technique for measuring lipid peroxidation or the quantity of TBARS in the primary rat hepatocyte 1.0 ml phosphate buffer and 2 ml 10% TCA were added to 1 mL liver homogenate and well mixed [62-63]. To precipitate proteins, the mixture was centrifuged at 3000 rpm for 10 minutes 2 ml supernatant was collected, and 0.5 mL 1% TBA was added, followed by one hour of heating at 95°C. The hepatic tissue was homogenized in ice-cold 0.15 M HCl (10%), and the absorbance at 532 nm was measured [63]. The absorbance was used to determine the concentration of TBARS, which was represented as nm of MDA per mg protein, using MDA as the standard.

4.2.4. Measurement of AST, ALT, and TNF-α

Instrumentation Laboratory assay kits and semi-automatic analyzer equipment were used to measure the AST and ALT activity. Primary hepatocyte cells were treated with EEP, GP, and the toxic agent CCl_4. The enzyme activities were tested immediately after the supernatant was withdrawn from the wells. The results were demonstrated in IU/L units. The TNF-α values were measured according to the supplied commercial kit.

4.2.5. CCl_4 cell viability assay

In vitro assay to identify hepatotoxicity effects of different doses of CCl_4. The hepatotoxic effects of CCl_4 treatment (0.5,10, 30, 50, 70 and 100 mM/ml) on primary hepatocyte cells were measured. CCl_4 showed a significant cytotoxic impact on primary hepatocytes. Normal control cells were incubated with DMEMA in DMSO (0.05% v/v) for 12 h. After 2 hours of exposure to a different toxic dose of CCl_4, we evaluated the LD_{50} value was calculated (15.57 mM), where more than 50% of the cells were dead.

4.2.6. Statistical Analysis

All data were given as means, ± SD (standard deviations). The triplicate test was performed for each treatment sample, and statistical analysis was performed using one-way analysis of variance (ANOVA) for AST, ALT, TNF-α, and lipid peroxidation assay. Statistical significance was considered for ($p<0.05$). Using the Microsoft Excel application, the percent (%) of cell viability, LD_{50} value. The GraphPad prism 9 from the Tukey test graphically represented AST, ALT, TNF-α, and MDA levels.

4.3. RESULTS:

4.3.1. Cell viability by MTT Assay

CCl_4 showed a significant cytotoxic impact on primary hepatocytes Fig. 1(a) with various types of concentrations. After 6 hours of exposure, the LD_{50} values 1(c) and 1(d) were calculated (15.5 mM), where more than 50% of the cells were dead. At low concentrations (15.57 mM), CCl_4 causes a 50% loss in cell viability. At greater concentrations (0.5-100 mM), fig. 1(a) showed a progressive decline in cell viability.Fig.1(b) showed that in MTT assay EEP, EEP +GP, and SLM effect on primary hepatocyte the cellular viability increased. Here, 1 (b) showed 40 mM CCl_4 highly reduced rat primary hepatocyte percentage of cellular viability. Here, 40 mm CCl4 treated with 100 μg/ml EEP treatment shows an increase in

cellular viability less than 100µg/ml EEP+100 µg/ml +40 mm CCl_4 and 100 µg/ml EEP+ 50 µg/ml GP+40 mM CCl_4. So, these results proved that EEP + GP treatment can increase hepatoprotection.

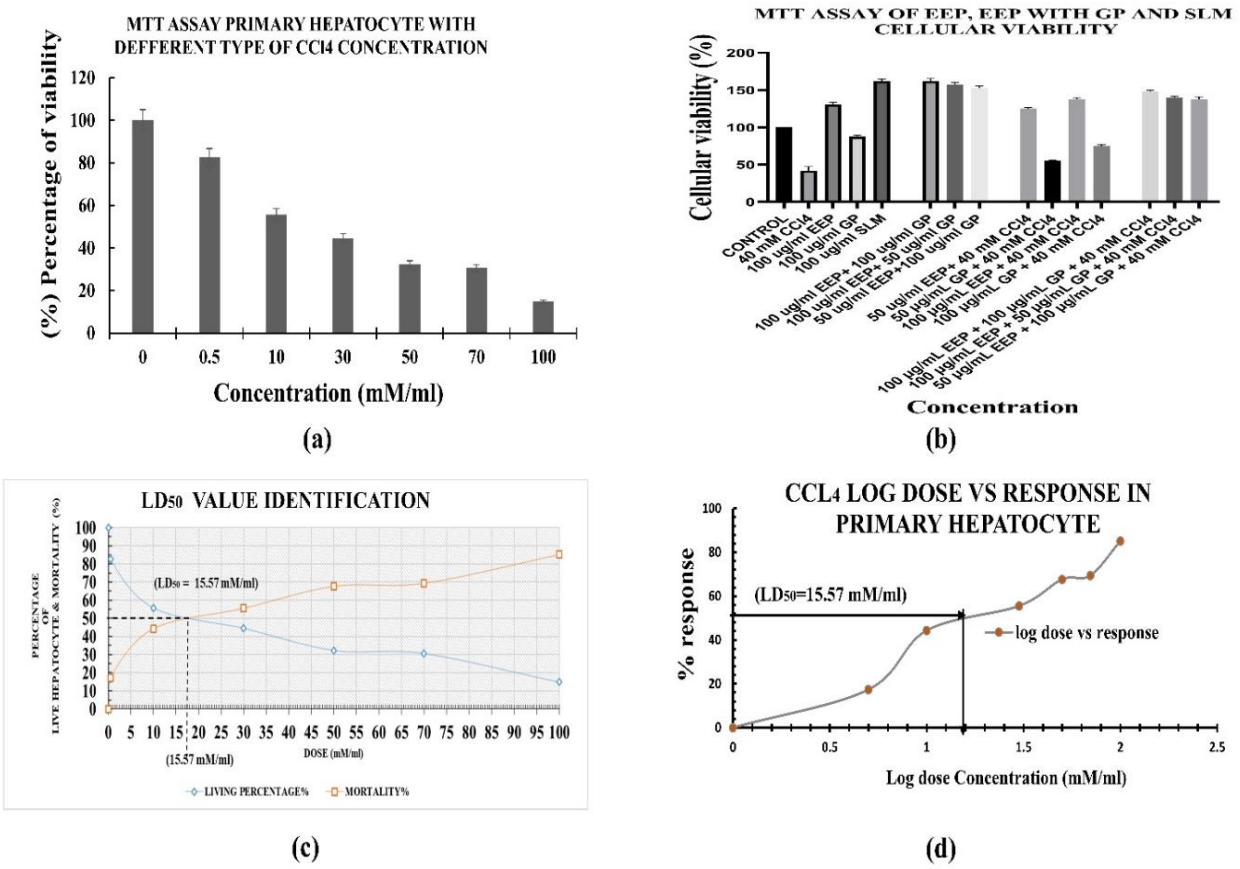

Figure 1. MTT assay showed the percentage of cell viability in primary hepatocyte of CCl_4 treatment Fig.1(a) decreased due to an increase in CCl_4 concentration; here, the control sample value was shown Fig. 1(a)100% cell viability was shown.Fig.1(b) In the MTT assay, the percentage of cellular viability increases EEP, EEP +GP, and SLM treatment on primary hepatocytes. Fig.1(c) & (d) percentage of live hepatocyte vs mortality percentage LD50 value identification. Fig.1(d) LD50 value of 50% cells were died 15.57 (mM/ml) concentration of CCl_4 treatment in log dose vs response in primary hepatocyte. However, due to CCl_4, the cellular survival ability is lost due to dose increase.

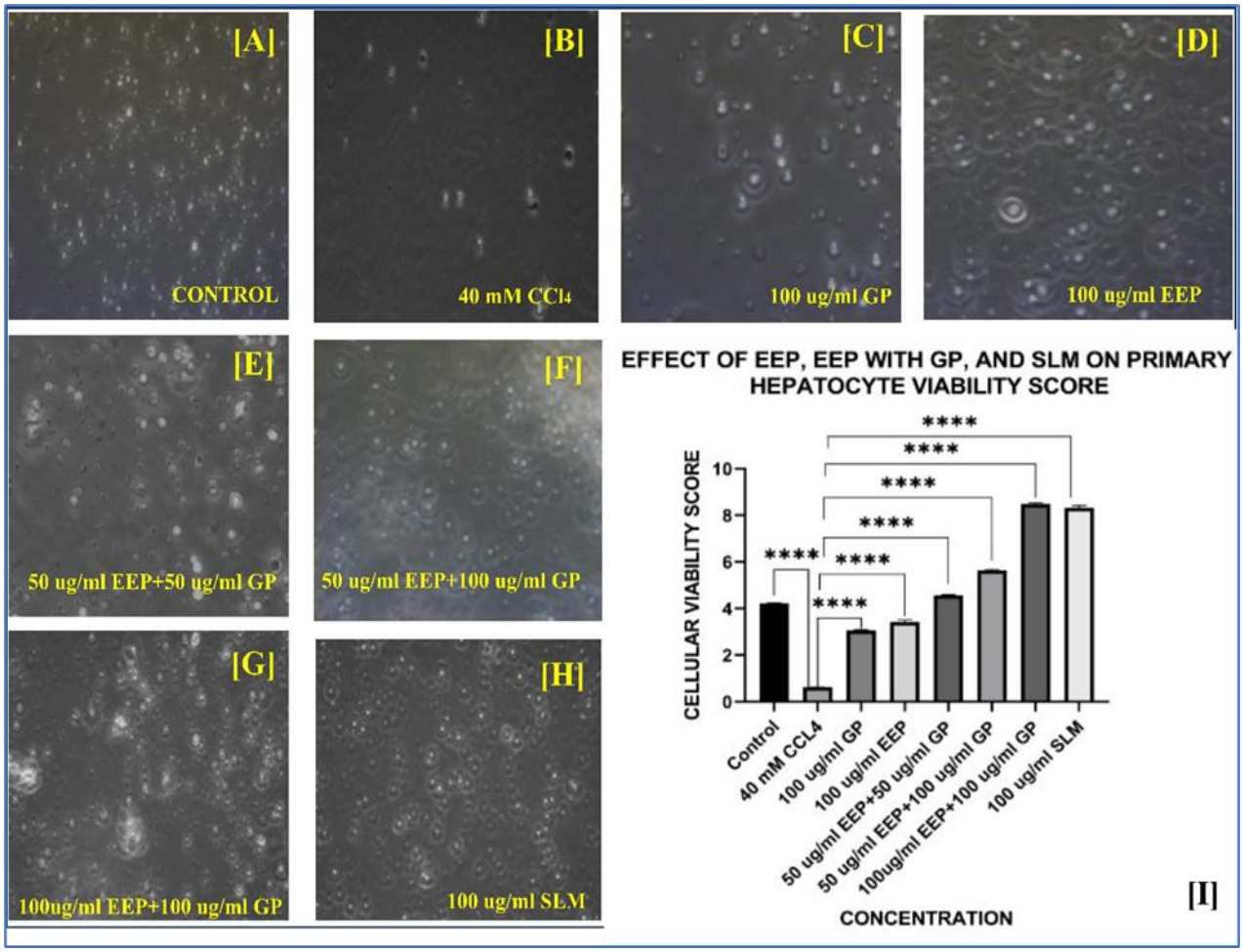

Figure 2. Rat primary hepatocytes were isolated and examined using a Carl Zeiss fluorescence microscope. Magnification = 10x. After 6 hours, the primary hepatocyte control group's (no treatment) cellular viability was observed in Fig.2(A). The proliferation of hepatocytes decreases with the treatment of a CCL_4 dose of 40 mM in Fig.2 [B]. 100 µg/ml GP treatment Fig. 2 [C] showed proliferation of primary hepatocyte EEP individual treatment (100 µg/ml) at Fig.2[D]. Fig.2[E], [F], [G] were treated EEE+GP in various concentrations and showed primary hepatocyte proliferation ****($p<0.0001$) Fig.2[I] & Standard treatment SLM Fig.2[H] (100 µg/ml) showed primary hepatocyte proliferation significantly.

4.3.2. Lipid Peroxidation Assay

Assay for Lipid Peroxidation Figure (3) shows the impact of CCl_4 on lipid peroxidation. This experiment revealed that increasing the concentration of CCl_4 increased MDA generation in primary hepatocytes. In addition, the data in Figure (3) showed that CCl_4-induced toxicity increased MDA levels, an indicator of lipid peroxidation. These findings suggest that CCl_4 causes LPO in response to oxidative stress.

4.3.3. CCl_4-induced cytotoxicity in Primary hepatocyte cells

The harmful effects of CCl_4 depended on both time and concentration. Significant variations in cell viability, AST, ALT, lipid peroxidation, and tumor necrosis factor (TNF-α) were seen compared to the control Fig. 3 & 4. Up to a 6-hour exposure duration, these variations were still significant control vs. CCl_4 treatment vs. treated test sample ($P < 0.05$).

4.3.4. CCL_4-induced toxicity in primary hepatocyte culture and EEP protectant

The toxic effects of CCl_4 are shown in Fig. 3 & 4. The release of AST, ALT, LPO (or MDA level), and TNF-α increased during the six h in primary hepatocyte cells exposed to CCl_4 and declined when EEP treatment without or with GP. The effect of CCl4-induced toxicity with various concentrations showed a dose-dependent toxicity increase in rat primary hepatocyte cell culture Fig. 1a. These changes remained significant up to an exposure time of 6 h ($P < 0.05$). The treatment group was given 25, 50, and 100 ug/ml earthworm powder and compared to the ccl4 toxic group in fig.3(a). Similarly, in Fig. 4(a) two concentrations of EEP + GP were taken at 50 and 100 µg/ml, and hepatoprotective activity of GP alone was shown. All treatments were again compared with the toxic group. Also compared with Fig. 3(a) and 4(a), our results showed that the result of the EEP + GP treatment group was more significant than that of EEP only 3(a).

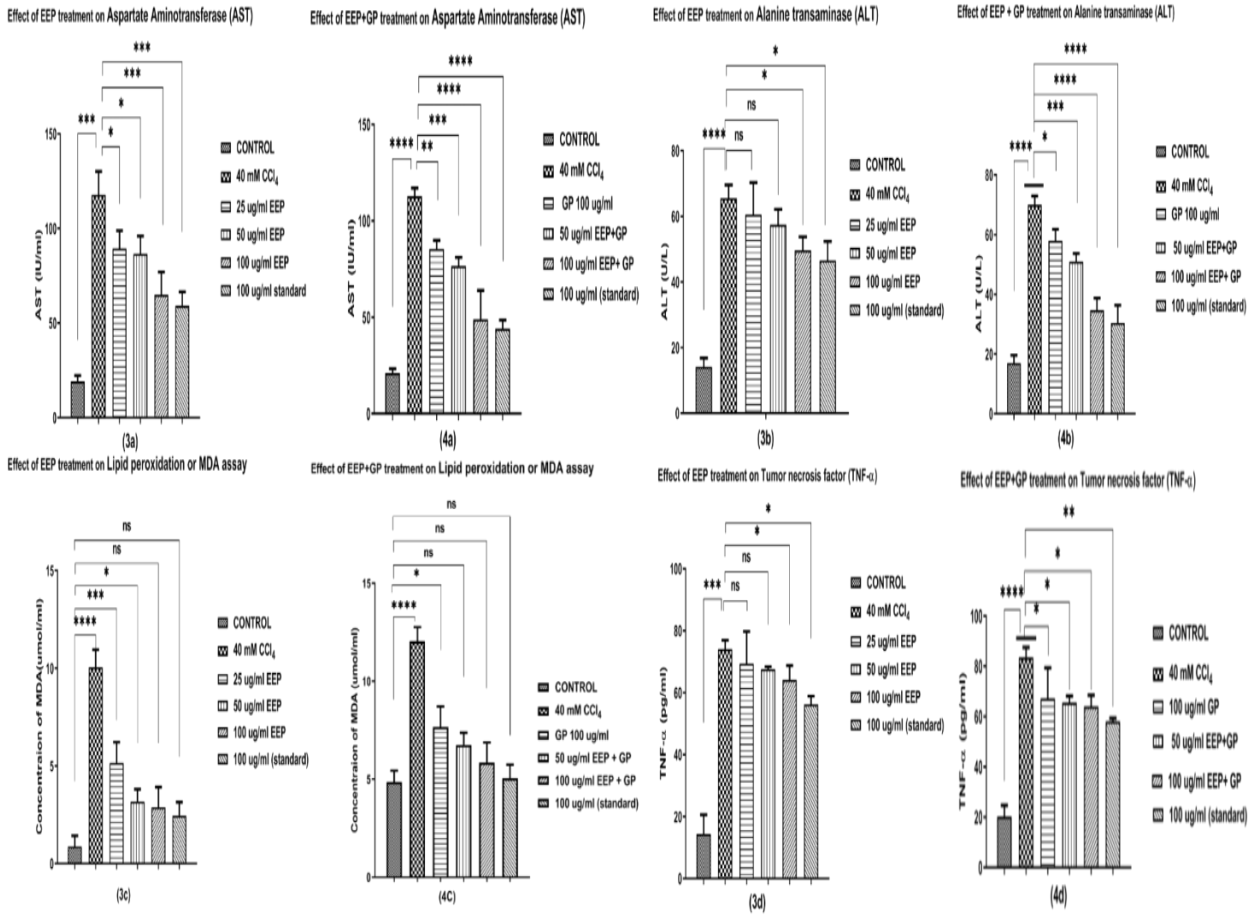

Figure: 3 & 4. In Fig. 3(a) & 3(b), EEP treatment at different doses was shown in our experiment; both AST and ALT 100 µg/ml showed p-values were significantly changed **($p<0.01$) & *($p<0.05$). Very Low MDA levels can be seen in the control group. Different concentrations (25, 50, 100 µg/ml) of EEP were evaluated for effect on the primary hepatocyte lipid in peroxidation or MDA assay. Here, Fig.3(c)control vs 25 µg/ml and 50 µg/ml show significant p-value change ***($p<0.001$) & *($p<0.05$), but 100 µg/ml and 100 µg/ml (standard) treatment were not significantly changed than 40 mM CCl_4 treatment ****($p<0.0001$). Fig.3(d). TNF-α p-value showed control vs 40 mM CCl_4 were significantly

changed ***($p<0.001$) in fig.3(d). In Fig. 3(d). CCl_4 vs. EEP treatment with (25 and 50 µg/ml) did not show TNF-α significantly change p-value ($p<0.05$), but 100 µg/ml EEP showed p-value was significantly change *($p<0.05$). In Fig. 4 (a) & (b), EEP + GP treatment shown in our experiment, both AST and ALT (50 and 100 µg/ml) showed p-value was significantly changed in ****($p<0.0001$) & *($p<0.05$). Very Low MDA levels were seen in the control group. The concentrations (50 and 100 µg/ml) of EEP + GP were evaluated for effect on the primary hepatocyte lipid peroxidation assay. Here, 40 mM CCl_4 vs control groups were shown significant p-value change ****($p<0.0001$). However, the EPP+GP (50 & 100 µg/ml) treatment did not significantly change the MDA level at 40 mM CCl_4 treatment. Fig.4(d). Tnf-α p-value showed control vs. CCl_4 was significantly changed ****($p<0.0001$) in fig.4(d). In Fig. 4(d). 40 mM CCl_4 vs EEP+GP of TNF-α were showed significantly change p-value *($p<0.05$) and **($p<0.01$). So, our result showed that EEP+GP treatment may increase hepatoprotectivity.

4.4. Dıscussıon

In assessment to the mid-nineteenth century, modern medicine significantly reduced mortality from several ailments. However, these new drugs that treat illnesses have various adverse side effects with long-term usage, and their improper use has triggered the emergence of diseases resistant to treatment with conventional medicines. Disease prevention and treatment have long been accomplished using medicinal plants. Through research and technology, it is necessary to find new bioactive compounds or biotechnological medications that are cheaper, safer, and have no side effects. Numerous studies have revealed that newly discovered proteins include a wide range of naturally occurring bioactive molecules with biological activity [47]. Multiple studies on novel medications and the efficacy of traditional medical practices have been published recently. The model of CCl_4-induced liver toxicity has been extensively researched to examine the adverse impacts of carbon tetrachloride (CCl_4) on the liver. Carbon tetrachloride (CCl_4) is a potent hepatotoxin that undergoes hepatic metabolism to generate highly reactive free radicals, thereby inducing oxidative stress,

inflammation, and hepatocellular injury[48]. This model is frequently employed to mimic the acute and chronic liver injury commonly observed in humans. Studies on CCl_4-induced liver toxicity have aided in understanding the causes of liver damage and discovering new therapeutics. Deterioration of hepatocellular damage, including hepatocyte necrosis, apoptosis, and vacuolar degeneration, results from CCl_4 administration. There is a correlation between this kind of injury and higher levels of liver enzymes like alanine transaminase (ALT) and aspartate transaminase (AST) in the bloodstream.Oxidative Stress CCl_4 metabolism generates reactive oxygen species (ROS) and causes lipid peroxidation, leading to oxidative stress in hepatocytes. Increased malondialdehyde (MDA), a marker of lipid peroxidation[49]. Herbal Medicines Many plant-derived substances protect against CCl4-induced liver damage. many studies found that antioxidant, anti-inflammatory, and anti-fibrotic milk thistle silymarin protects liver cells from CCl4 damage [50]. There are several studies have been done on CCl_4 liver damage. In our studies, the first experiment, the 1st set of primary hepatocytes divided into six groups were treated with different concentrations (0, 0.5, 10, 30, 50, 70, 100 mM) of CCl_4 MTT assay to identify cellular viability and LD_{50} was calculated in fig.1(a, c & d). The second experiment, fig.1(b), constitutes the MTT assay with the 2^{nd} set of primary hepatocytes divided into four sequences with different concentration treatments to identify the effect of control, 40 mM CCl_4, EEP, GP, EEP+GP, and SLM. Here, we showed 40 mM CCl_4 reduced percent of cellular viability in the primary hepatocyte. The administration of 50 or 100 µg/ml of EEP+GP has demonstrated a significant enhancement in primary hepatocyte proliferation compared to the only administration of EEP. Our findings reveal that CCl_4 highly inhibited the proliferation of the primary hepatocyte cell culture in the laboratory model after 6 hr of incubation. EEP protein lower dose of 25 µg/ml in CCl4 demonstrated changed levels of MDA as well as cell cytotoxicity, loss of cell viability, and leak of liver enzymes into the culture media, but the 50 to 100 µg/ml showed a positive hepatoprotective effect. The current study provides various types of disease treatment evidence for the conventional usage of protein derived from earthworm extract [14]. As a result, the extracts' ability to serve as antioxidants and as hepatoprotection against CCl_4 liver-damaging effects were evaluated. In addition, numerous studies explained the association between liver damage and oxidative stress. The cytotoxic effect of CCl_4 on primary hepatocytes was investigated in our experiment. Our findings reveal that CCl_4 highly

inhibited the proliferation of the primary hepatocyte cell culture in the laboratory model after 6 hr of incubation. EEP protein lower dose of 25 µg/ml in CCl_4 demonstrated changed levels of MDA as well as cell cytotoxicity, loss of cell viability, and leak of liver enzymes into the culture media, but the 50 to 100 µg/ml showed a positive hepatoprotective effect. Reactive oxygen species (ROS) injure the liver by causing hepatocyte malfunction and apoptosis, Kupffer cell infiltration, and activation of hepatic stellate cells. The endoplasmic reticulum and the electron transport chain in the mitochondria of hepatocytes are the primary sources of ROS. Although it had less efficacy than SLM, the EEP demonstrated a dose-dependent percentage inhibition on the measured free radicals. The inhibition could become more effective as the increase of concentration. The current investigation revealed that concomitant administration of goat milk and EEP, together with CCl_4, helped lower the degree of primary hepatocyte toxicity; the results were comparable to those of the reference treatment, silymarin. Goat milk and EEP administration decreased oxidative stress, enzymatic activity, and inflammation. Superoxide radicals are the major free radicals for oxidative stress because they create other free radicals; our studies in-vitro assay EEP showed decreased development of superoxide free radicals by lipid peroxidation assay. In addition, our in-vitro assay showed that natural products protein EEP can protect the liver. Mitochondria and their impacts on hepatic intracellular cell activity in the context of hepatoprotective qualities might reveal possible therapeutic benefits of particular drugs or therapies. CCl_4 is often used to generate liver damage in experiments. Reactive oxygen species from CCl_4 produce oxidative stress and liver damage [51]. Hepatocyte damage—liver cell injury or impairment—can affect mitochondria, the energy-producing organelles in cells. Cellular respiration generates ATP from mitochondria. Oxidative stress may damage mitochondria and cause cell death. Hepatocyte injury may damage mitochondrial membranes, especially the outer membrane. Increased permeability allows pro-apoptotic substances from the mitochondria to enter the cytoplasm [52]. As a consequence, apoptosis may occur. Hepatocyte injury may compromise mitochondrial energy generation, structural integrity, and critical processes. Mitochondrial changes cause liver cell malfunction and may affect liver health and disease [53]. The mitochondria and impairing function of hepatic intracellular cells were evaluated for their hepatoprotective properties using the CCl_4-induced liver toxicity technique. Because of its lipophilicity character, CCl_4 spreads quickly across tissues and through cell membranes [54].

CCl_4 induces cellular toxicity and inhibits cell proliferation. After 12 hours of culture, we found that CCl_4 is cytotoxic to rat primary hepatocytes, with an LD_{50} of 15.57 mM/ml fig: 1(c, d). Our findings also show that the initial concentration of CCl_4 (0.5 mM/ml) did not show higher cytotoxicity. This eventually results in a rise in bile acid levels, mainly *in-vivo* system, higher levels of which frequently boost liver biomarker enzymes such as AST, ALT, ALP, and total bilirubin, and reduce total protein levels in the blood plasma, cause liver damage via oxidative stress in the animal model. Therefore, only the biomarker enzymes AST, ALT, MDA, and TNF-α were examined in the current investigation. In the 3rd experiment, the 3rd set of isolated primary hepatocytes was classified into six groups, namely control, 40 mM CCl_4, 25 ug/ml EEP, 50 µg/ml EEP, 100 µg/ml EEP, and 100 µg/ml SLM standard. Compared to the standard medication silymarin, the present study in hepatoprotective activity results show that EEP has more hepatoprotective activity. When compared to the toxic group (CCl_4 treated 40 mM/ml), the EEP significantly ($p<0.05$) reduced the elevated biomarker enzyme levels AST, ALT, and MDA levels. There could be a connection between EEP with antioxidant and hepatoprotective properties. We used lipid peroxidation to investigate the probable role of MDA formation in CCl4-induced toxicity. The findings of this experiment revealed that CCl_4 treatment significantly raises ($p<0.05$) MDA levels in primary hepatocyte culture media and suggests that oxidative stress plays an essential role in CCl_4-induced toxicity. The reactive oxygen species (ROS) produced by CCl_4 are well-known. The inflammatory cytokine TNF-α here increased due to the 40 mM CCL4 treatment. But EEP treatment here showed 50 and 100 ug/ml significant hepatocyte protection activity. These set also demonstrated p-value significantly change respectably in (Fig.3) a, b, c, d *($p<0.05$) **($p<0.01$) ***($p<0.001$). The inflammatory response was examined to determine the potential molecular cause, and the oxidative response was investigated. According to reports, during various oxidative stress conditions, the inflammatory response is a key step that causes organ damage [55]. As previously observed in multiple study papers, CCl_4 treatment caused acute liver damage strongly related to inflammation via increasing proinflammatory cytokine release [14]. TNF-α, an inflammatory cytokine, was shown to be significantly increased in CCl4-treated generated primary hepatocyte culture in vitro, representing a main cause of liver cellular damage. It is remarkable that the administration of EEP+GP considerably decreased the release of AST,

ALT, and TNF-α inflammatory cytokine, indicating that it could reduce CCl_4-induced liver damage. Here, only 100 ug/ml GP has also shown hepatoprotection but less than the EEP and EEP+GP treatment in our experiment. 4^{th} set of isolated primary hepatocytes was divided into six groups: control, 40 mM ccl4, GP 100 ug/ml, 50 ug/ml EEP+ 100 ug/ml GP, and 100 ug/ml EEP+ 100 ug/ml GP and 100 ug/ml SLM (Fig 4) a, b, c, d. These set also demonstrated p-value significantly change respectably *($p<0.05$) **($p<0.01$) ***($p<0.001$) and ****($p<0.0001$). If we look at the NF-kB pathway, we can see that oxidative stress raises SOD, CAT, LPO, MDA, and eventually IL6, IL-1β, and TNF-α, which is relevant to our experiment [41]. The NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a signalling pathway that regulates immune responses, inflammation, cell survival, and other biological processes. It plays a crucial role in activating genes that control the production of various inflammatory mediators, including TNF-α (tumor necrosis factor-alpha) [56]. Oxidative stress is an imbalance between reactive oxygen species (ROS) production and the body's antioxidant defence mechanisms. ROS can cause damage to cellular components, including lipids, proteins, and DNA. When oxidative stress occurs, it can activate the NF-kB pathway, leading to the upregulation of various genes involved in inflammation and immune responses [57]. One of the markers of lipid peroxidation, a process that occurs under oxidative stress, is the increase in lipid peroxides or lipid peroxidation products. Malondialdehyde (MDA) is a commonly used marker to assess lipid peroxidation. Elevated levels of MDA indicate increased lipid peroxidation and oxidative damage to cell membranes. In the context of our experiment, oxidative stress-induced elevation in LPO or MDA levels suggests an increase in lipid peroxidation and oxidative damage in the primary hepatocyte for CCl4-induced. Additionally, 40 mM CCl_4 increased the production of TNF-α in response to oxidative stress, indicating the activation of the NF-kB pathway, which is relevant to the inflammatory responses studied in our experiment. In vitro studies show that CCl_4 exposure produces superoxide, hydrogen peroxide, and hydroxyl radicals in epithelial cells [58]. The activation of a free radical is created by an imbalanced metabolite, such as on unsaturated lipids, which initiates the process of lipid peroxidation, and the subsequent response is halted by the formation of lipid breakdown products, aldehydes, and malondialdehyde [59][60-63]. In our studies, the hepatoprotective involves primary hepatocyte growth in a culture medium and the restoration of hepatic AST, ALT, MDA, and

TNF-α levels Fig.3 & 4 with treatment EEP and EEP+GP. The combination treatment of EEP+GP here showed better than the only EEP treatment on the primary hepatocyte due to 40 mM CCl4. However, a more accurate application of this bioactive protective agent would depend on a better understanding of the underlying mechanisms behind only EEP or a combination EEP+GP hepatoprotective effect.

Conclusıon

In conclusion, the present study showed the potential hepatoprotective effects of both EEP and EEP+GP against CCl_4-induced liver damage where the addition of GP enhances the hepatoprotective action by reducing inflammation and oxidative stress. EEP+GP probably increases the liver regeneration process or cellular proliferation. EEP and GP combination overcome high levels of toxicity and oxidative stress. This bioactive EEP, together with GP, may thus be investigated as a possible bio-therapeutic agent for human disorders, particularly for the treatment of acute or chronic liver injury. The bioactive protein has several potential uses, particularly in the food and pharmaceutical industries.

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CHAPTER V

Natural Remedies for Hepatic Diseases

5. Materials

DCA, CDCA, Cholic acid and tolbutamide, as internal standard substances (IS) used in the study, were provided by Sigma. Acetonitrile and methanol were HPLC and LC MS/SM-grade and purchased from Merck (Darmstadt, Germany). Phosphoric acid, potassium phosphate monobasic anhydrous, and formic acid were supplied from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade water was used to prepare mobile phase solutions. All solutions were filtered through a 0.45 μm (pore size) nylon membrane filter and vacuum degassed before use. HPLC analyses were perforcxmed using a Schimadzu UFLC chromatographic system (Schimadzu, Kyoto, Japan). CYP2E1 ELISA Kit Rat Cytochrome P450 2E1 mybiosource (CYP2E1) ELISA Kit (Catalog mbs2019931), IL-4 Rat ELISA Kit (Cat #BMS628 Invitrogen), Rat Stat3 (Sandwich Elisa) Elisa Kit - Ls-F336478, Trypan Blue Solution, 0.4% (cat no. 15250061), Pierce™ Modified Lowry Protein Assay Kit (cat no. 11240), Amicon® Ultra Centrifugal Filter, 3 kDa MWCO sigma Aldrich (cat no. UFC5003), Amicon® Ultra Centrifugal Filter, 50 kDa MWCO sigma Aldrich (cat no. UFC905008). DCA (Deoxycholic acid) and CDCA (Chenodeoxycholic acid) purchased from sigma-aldrich

5. 1 Method

5.1 Earthworm protein powder preparation treatment for EtOH-induced hepatotoxicity

To remove dirt, lab-raised Indian earthworms (*Metaphire posthuma*) were washed with tap water and kept in water overnight. After sonication to clear mucus, the earthworms were homogenized. The sediment was collected by sonicating and centrifuging the pureed solution at 4,000 rpm at 4 °C. Protein was extracted by ammonium sulfate precipitation (salting-out), diluted in PBS, and loaded onto centrifugal units with a 50-kDa nominal molecular weight cut-off (MWCO). The filtrate from the first ultracentrifugation was collected. The fluid retained by the filter from the second ultracentrifugation with a 3-kDa MWCO was gathered

and prepared for analysis [1]. The extra salt is then removed by dialysis across the membrane. The protein suspension was then collected and prepared for freeze-drying, which was then powdered and fed to rats of various weights. Bradford assay, a quick and sensitive method, was used to determine the protein concentrations [2].

5.2 Earthworm protein powder preparation treatment for CCl4-induced hepatotoxicity:

Metaphire posthumous Indian earthworms weighing 2 kg were purchased from a vermicomposting factory in Lila Agrotech Pvt Ltd, Madhyamgram, West Bengal, India. Earthworms were kept alive for a week in humus-enriched soil with a constant supply of nutrients. Earthworms fully developed and between 15 and 20 cm long were chosen to prepare the crude extract. 500 g of adult earthworms salting out process extract pallet formation due to centrifuge (12,000x g) for 15 minutes at 4 °C. Then the homogenized autolyzed for 4 hours at 50°C in a 50 mM phosphate buffer solution with a pH of 6.0 and 0.2 percent sodium azide as a bacteriostatic[2][3]. Then, the Centriprep® Centrifugal Filter Device, which was a disposable ultrafiltration device, was used for purification to remove proteins below 10 kDa. The aim of taking protein below 10 kda is to have high therapeutic activity of the low protein[4]. Dialysis membrane was performed after the freeze-dried earthworm extract in 50 mM phosphate buffer at a pH of 6.0 in 50 ml. The protein concentration passed through membrane dialysis was calculated. Biological samples were concentrated, desalted, and filtered in a volume range of 2–15 mL [5]. The suspension-type solution was then freeze-dried to make a powder. The Lowry protein assay determined the total protein content [6]. This lyophilized powder was stocked and evaluated for hepatoprotective activity on rats (treatment group respectably 50, 100 & 200 mg per kg body weight). Dialysis membrane was performed after the freeze-dried earthworm extract in 50 mM phosphate buffer at a pH of 6.0 in 50 ml.

5.3 Acute toxicity study of earthworm extract

5.3.1 Acute oral toxicity (single dose) test

In this study, male and female rat were divided into three groups, with six rat in each group viz. Group – 1: Vehicle; Group – 2: 2000 mg/kg (Male); Group – 2: 2000 mg/kg (Female). Prior to the start of the experiment, the rats were fasted for 18 hours but were allowed access to water until 1 hour before the treatments began. The rats were weighed and orally administered with either a vehicle (distilled water) at a volume of 10 mL/kg body weight or the highest dose of the MP dissolved in water (2000 mg/kg body weight). The rats were then monitored for mortality and signs of toxicity at specific time intervals (15, 30, 60, 120, 180, and 240 minutes) and once daily for a period of 14 days. The experimental protocol followed guideline 423 with slight modifications as outlined by the OECD/OCDE in 2002 [7]. Malone and Robichaud (1962) [8] observed that there were observable physical and behavioral changes that indicated either stimulation or depression of the central nervous system (CNS), along with the presence of pallor, cyanosis, and alterations in urine and feces when a drug is administered. These changes were observed shortly after the initiation of therapy. The animals were housed in polypropylene cages and provided with unlimited access to feed and drink for a duration of one hour following the administration of the treatments. Upon completion of the 14-day period, the animals underwent a subsequent weighing procedure to ascertain any alterations in weight throughout the course of the experiment. Subsequently, the animals were euthanized through intraperitoneal administration of 60 mg/kg ketamine and 10 mg/kg xylazine. Following euthanasia, the primary organs, namely the lung, heart, liver, and kidneys, were extracted and subjected to macroscopic examination to identify any observable changes in anatomy, color, and size. Furthermore, the relative weights of these organs were determined according to de Carvalho, M. S. et al., 2024 [9].

5.3.2 Subacute toxicity (repeated doses)

The evaluation of subacute toxicity involved the administration of MP to rats (n = 6/group) by oral exposure once daily for a period of 28 days. The dosages administered were 500, 1000, and 1500 mg/kg/day. This experimental design maintained to the guidelines outlined in the OECD guideline 407, which was established in 2008 (OECD/OCDE, 2008) [10]. Body weight of the animals were recorded in a weekly basis. The clinical presentation of toxicity was assessed by monitoring several symptoms and indications, including alterations in the integumentary system (skin, hair, eyes, and mucous membranes), as well as the circulatory, gastrointestinal, respiratory, central nervous system, peripheral nervous system, and general behavioral manifestations. On the twenty-eighth day of the experimental period, the animals underwent anesthesia using ketamine and xylazine at doses of 100 and 10 mg/kg intraperitoneally, respectively, in order to obtain biological samples. Following this, a sample of blood was obtained from the retro-orbital plexus and subsequently submitted for examination in order to assess its hematological and biochemical properties. Subsequently, the animals were subjected to euthanasia by administering xylazine/ketamine (10/100 mg/kg intraperitoneally) to induce severe anesthesia, facilitating the extraction of specific organs (liver, kidneys, heart, and lungs) for further anatomical and histological evaluations.

5.3.3 Biochemical and hematological analyses

The hematological analysis was conducted on the blood samples obtained from anesthetized animals, that were treated with different doses of MP (500, 1000, and 1500 mg/kg/day). The study utilized a blood collection tube (Vacutainer®, USA) for storage of blood samples. The collected samples were subsequently analyzed for hematocrit (Ht), hemoglobin (Hb), platelet count, red blood cell count, total leukocyte count, neutrophil count, lymphocyte count, monocyte count, eosinophil count, and basophil count using an automated cell Medonic M51 (Boule Medical AB, Sweden). Blood samples were obtained using a vacuum-sealed collection device with a volume of 4 mL (Vacutainer®, USA), which was equipped with an

activated silica gel clot activator weighing 1.2 g. Subsequently, the samples underwent processing, resulting in the acquisition of serum for the purpose of conducting biochemical analysis. This analysis comprised the measurement of glucose, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, total cholesterol, and triglycerides. The measurements were carried out using the automatic analyzer Selectra Pro S (ELITech Group, Netherlands).

5.3.4 Determination of relative organ weights, macroscopic and histopathological analysis

Following the blood collection procedure, the rats underwent the removal, weighing, and examination of their major organs, including the heart, lung, liver, and kidneys. Additionally, the internal cavities in which these organs were located were also inspected for potential abnormalities. Subsequently, the relative weight of the organs was also measured using [(organ weight/body weight) × 100]. Following this, sections of each organ were surgically removed and preserved in a 4% paraformaldehyde solution for further histological examination. The organs were subjected to a histological processing protocol according to de Carvalho, M. S. et al. [11] with slight modifiations. This involved a series of steps, including dehydration in progressively higher concentrations of ethanol, clarification in xylene, embedding in paraffin, and finally sectioning the organs into 6 μm slices using a cryostat, Leica CM 1520 (Leica Biosystems, Germany). Subsequently, slides were made and subjected to staining with hematoxylin and eosin. These slides were then examined using phase contrast microscope (EVOS XL Core Imaging System, ThermoFisher, USA) to assess the presence or absence of edema, cell infiltration, vascularization, tissue organization, and necrosis, respectively.

5.4 Experimental Design for Ethanol (EtOH) induced liver injury

Water and standard laboratory pellets were made available to all the animals without any restrictions. Housing of the animals was maintained under pathogen-free conditions with a

12-hour light/dark cycle, a constant temperature of 25 ± 2 °C, and approximately 60% humidity. The first week was spent acclimatizing the rats. All animals had a body weight within 150 to 155 g. The animals were randomly divided into the following six groups:

Group-I (Normal treatment for 14 days),

Group II (Ethanol (EtOH) 4 g/kg body weight for 14 days oral administration),

Group III (Ethanol (EtOH) 4g/kg + Goat milk (GM) 10mL/kg body weight for 14 days oral administration),

Group IV (Ethanol (EtOH) 4g/kg + MP 50mG/kg body weight for 14 days oral administration),

Group V (Ethanol (EtOH) 4g/kg + MP 50 mg/kg for 14 days oral administration + GM 10 ml/kg/day body weight for 14 days oral administration),

Group VI (Ethanol (EtOH) 4g/kg + MP 100 mg/kg for 14 days oral administration + GM 10 ml/kg/day body weight for 14 days oral administration).

Recently, a few of study have claimed that goat milk at a dose of 10 ml /kg of body weight had hepatoprotective action. Therefore, throughout the course of this research, goat milk was used as a suitable as a vehicle [12].

5.5 CCl_4-induce fibrosis for experimental design in animal model

A previously established technique was used to create CCl_4-induced liver fibrosis in rats[15]. CCl_4 reconstituted in olive oil (0.5 ml/kg; 1:1 v/v, IP) was administered twice a week for 12 weeks [13]. After 12 weeks of CCl_4 injection, six rats were chosen randomly and sacrificed under Diethyl ether anesthesia, and their blood was taken after anesthesia via the Retro orbital plex. Then, they were collected and separated by centrifugation for 10 min at 4000 rpm in a cold centrifuge (4^oC). The rats were divided into six groups of (n=12) rats each, as well as a control group [14].

The control group (GROUP-I) got normal food treatment.

The disease control group (GROUP II) got 12 weeks of IP CCl_4 injection, weekly twice.

Treatment MP groups (Group-III, Group-IV, and Group-V) got 12 weeks of IP injection of CCl_4(weekly two times) and MP dose (50, 100, and 200 mg/kg) for twelve weeks.

The standard group (Group VI) administered a CCl_4 IP injection, and Ursodeoxycholic acid (200 mg/kg) for 12 weeks [15].

5.6.1 In-vitro test

5.6.1.1 In vitro cell viability Assay of primary hepatocytes for EtOH hepatoxicity

Primary hepatocytes were isolated using a recombinant enzymatic method, with slight modifications to the perfusion technique adopted from our previously published work [2][16]. Anesthesia was conducted using diethyl ether. After opening the abdomen, the liver was perfused through the portal vein with a Ca^{2+} and enzyme-free buffer (NaCl 0.142 mM, KCl 0.0067 mM, HEPES 0.01 mM, oxygenated for 10 minutes, pH 7.4) for 12 to 15 minutes at a flow rate of 2.5 to 3 ml/min. This was followed by reperfusion for 7–8 minutes with the same buffer containing collagenase (1.6×10^{-9} mg/ml, 630 units/mg solid, at 37°C and pH 7.6) at the same flow rate. After disruption of the tissue, filtration, and cold centrifugation (4°C, 200 rpm, three times), the hepatocytes were obtained. The viability of the hepatocytes was evaluated using the trypan blue exclusion assay [2][17].

5.6.2. In-vivo test

5.6.2.1 Blood sample preparation for CCl_4 & EtOH-induced hepatotoxicity

Blood samples were collected from the tail vein approximately 24 hours after the final drug treatment. These samples were then centrifuged at 4,000 g for 15 minutes to obtain serum. This serum was subsequently used to estimate the levels of serum marker enzymes related to liver function and anti-oxidant activity [18]. After blood samples are collected, they are stored appropriately for testing.

5.6.2.2 Assay of Hepatic Enzymes and Serum biochemical markers for EtOH hepatotoxicity

Following the manufacturer's instructions, cholesterol, AST/ALT ratio, and albumin was measured using Selectra Pro S (ELITech Group, Netherlands) autoanalyzer. The spectrophotometric analysis using Spectrophotometer M5 (Molecular Devices, USA) was used to assess the serum Glutathione (GSH), CYP-2E1 and catalase (CAT) concentration and ELISA of serum plasma biomarkers like TNF-α and IL-6 was performed according to the kit instructions.

5.6.2.3 Blood sample analysis of liver function, oxidative stress, antioxidant and inflammatory cytokine for CCl_4-induced hepatotoxicity

The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, and Bilirubin were measured in rat blood plasma. The amounts of lipid peroxidation (measured as malondialdehyde (MDA), and GSH activity were all determined using rat blood plasma. The different biochemical parameters were measured using commercially available kits according to the manufacturer's procedures. The enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of cytokines (IL-4, IL-6, TNF-α, and STAT-3) was performed according to the kit instructions.

5.6.2.4 Enzyme-linked immunosorbent assay (ELISA) method for sample quantification

All samples were centrifuged at 2000 g for 10 minutes at 4 °C and kept at -80 for future use. In the 96-well ELISA plate, we added 100 ul of standard diluents to each blank control well, 100 ul of sample, and 100 ul of standard diluents to each standard well [19]. To each well, we added 50 ul of the diluted antibody. These were wrapped in sealing film and incubated at room temperature (18°C-25°C) for two hours. After adding 300 ul of washing solution to each well, the liquid in each well was removed after one minute. This process washes the plate three times. It was dried on filter paper. Each well received 100 ul of streptavidin-horseradish peroxidase wrapped with seal film and kept at room temperature for 20 minutes.

After adding 300 ul of washing solution to each well, the liquid in each well was discarded after one minute. Three times, this was done. For the color development, we added 100 ul of TMB Chromogen Solution to each well and incubated them at room temperature for 10-15 minutes. The reaction was rapidly stopped by adding 100 ul of termination solution to each well. After stopping the reaction, the plate was read at a suitable detection wavelength according to provided commercial kit. A standard curve was created, and the standard concentrations in the samples were assessed using the optical density (OD) value [19].

5.6.2.5 End point PCR Qualitative Identification and expression NRF-2, AKT-1, α-SMA & BAX in MP and CCl_4 induced hepatotoxicity and primer design

mRNAs were extracted from liver tissue using a Trizinxp RNA Isolation Kit (GR1004T) (GCC biotech) by the manufacturer's instructions. The mRNA amount was determined using a spectrophotometer at 260 nm. Then, using a First Strand cDNA synthesis kit, RNase H minus 200 ug of mRNA was reverse-transcribed into cDNA (G4645). Before usage, the cDNA samples were kept at 80°C. A reaction mixture including 10 ul of PCR was amplified to a final concentration of 300 nM using Hi-Prime Taq DNA Polymerase (G7116), 2 or 3 ul of cDNA, and primers in an MJ Research mini cycle. Primer Express software was used to build primers based on primers (Applied Biosystems). Records of the primer sequences are kept. The following amplification cycles were used for the α-SMA, BAX, AKT, NRF2, and GAPDH (housekeeping gene). 10 minutes at 95°C, 40 cycles of 1 minute each at 95 °C, 56 °C, and 70°C, and finally 7 minutes at 72 °C. The amplification products were electrophoresis in 3 % agarose gels at 80 V to separate them, and an ethidium bromide stain was used to make them visible in the gel dock.The forward and reverse primer sequences were used to determine GAPDH mRNA expression in liver damage following paracetamol intoxication. All primers were designed from the National Center for Biotechnology Information (nih.gov) https://www.ncbi.nlm.nih.gov/ and All the primer design forward and reverse primer given below:

NRF2F:5'ACACAGCATAGCCCATCTCGT3'R:5'ACCAACCTGGATGAGCGACAC3',
ASMAF:5'GCTATGCTCTGCCTCATGCC3'R:5'CACGCTCAGCAGTAGTCAGAA3'
AKT1 F:5'GTGGCAAGATGTGTATGAG-3' R:5'CTGGCTGAGTAGGAGAAC-3',
BAX F:5`AGGGTGGCTGGGAAGGC-3` R:5`TGAGCGAGGGCGGTGAGG-3`

5.6.2.6 Body and Liver Weight Calculation for EtOH-induced hepatotoxicity

The body weight of each rat from a distinct experimental group was assessed. Calculating the liver restitution weight also allowed for the study of liver-mass increase and regeneration. Then, the total number of fatalities was observed in each group [20]. The percent of increase formula given below.

$$\% \text{ increase} = 100 \times \frac{(\text{final} - \text{initial})}{|\text{initial}|}$$

5.6.2.7 Bodyweight measurement for CCl_4-induced liver damage

All animal in each group had their baseline body weight recorded on the first day of treatment and every week during the treatment period. Weight gain was measured and reported as a percentage of total weight gain. Additionally, total animal mortality was observed at 12 weeks [21].

5.6.2.8 Histopathology of liver EtOH induce liver toxicity

Hepatic samples from each group were examined using the Evos XL microscope. The samples were stained with hematoxylin-eosin after being embedded in wax and fixed with a 10% formaldehyde solution in an isotonic solution. The criteria used to examine morphological anomalies included apoptosis, necrosis, and congestive hepatopathy. (absence, + mild, ++ moderate, and +++ severe).

5.6.2.9Histopathology CCl_4 induced hepatotoxicity

The histological procedure was employed on rat liver pre-fixed in 10 % formaldehyde solution (formalin). To eliminate all water from the tissue and replace it with alcohol, each

organ withdrawn from the formalin was submerged in a succession of ethanol solutions. The tissues were waxed into a cassette, microtomed, and flame-burned before being rinsed under running water, immersed in an alkaline solution, and stained with hematoxylin and eosin staining 1%. After putting the slices in the mounting medium, the sections were examined 20X under an EVOS XL microscope.

5.6.2.10 HPLC analysis for DCA and CDCA effect of MP, GM, and EtOH induce hepatotoxicity

For quantification of DCA and CDCA in plasma a LC-20A Dvp pump (Shimadzu Kyoto, Japan), with an UV absorbance detector and a Shimadzu SDP-20 Avp system in the HPLC was used in this experiment. Analysis was performed using a Luna 5m C18 column (250 mm × 4.6 mm, 5 mm; Phenomenex, Torrance, CA, USA) along with 1 ml/min flow rate. The isocratic mobile phase was composed of a mixture of 40% water, 60% acetonitrile, and 0.1 % formic acid. The wavelength of the UV detector was adjusted to 210 nm. The injection volume was 20 µl. The identified HPLC technique was according to the ICH guidelines.

5.6.2.11 Limits of detection and quantification, as well as linearity for HPLC

HPLC analysis of a blank plasma sample with IS and 5 various concentrations of tolbutamide (0.001–0.025 µg/mL) was used to create calibration plots. For quantification, the sample was tested in HPLC, and the retention time was compared to the standard tolbutamide retention time. The DCA and CDCA concentration was calculated by substituting the peak area of the sample solution into the calibration equation of the standard curve.

5.6.2.12 Method validation for HPLC of DCA & CDCA

The methodology was validated using quality control samples from the calibration curve at seven concentration levels (50.125, 100.25, 300.50, 800.00, 2150.00, 3200.00, 5000.00 µg/ml). To measure the intraassay accuracy and precision, this technique was done four times over four days. Six samples of the limit of ditection (LOD) and limit of quantification (LOQ) were treated at 4 °C and compared to newly produced calibration

standards to test post-preparation stability. Six sample LOD, LOQ were analysed after three freeze-thaw cycles and compared to a newly produced calibration curve to assess freeze-thaw stability. The stability of DCA and CDCA in plasma samples at room temperature was assessed by storing in of LOD, LOQ for 6h at room temperature, processing, and analysis against newly processed calibration curve standards [22].

5.7 Cholic acid quantification by LC ESI-MS/MS analysis for MP and CCl_4 induced hepatotoxicity:

5.7.1 Plasma Extraction Procedure LC ESI-MS/MS for MP and CCl4-induced hepatotoxicity

100 µl of untreated plasma was taken and precipitated with 400 µl of MeCN containing 1000ng/ml Tolbutamide (IS) and vortex for 10 min, followed by Centrifugation for 10 min. At 12,000 rpm at -20℃. 300µl supernatant was taken and transferred to autosampler vials for injection.

5.7.2 Preparation of Tolbutamide (IS TD) stock solution (W/V)

Tolbutamide was accurately weighed and completely dissolved in 1.0 mL dimethyl sulfoxide, then mixed and vortexed. As a result, the final concentration of Tolbutamide was 1 mg/ml. The standard solutions were used to make Tolbutamide intermediate concentrations. It was kept at 2-8°C in the refrigerator.

5.7.3 Quantification of cholic Acids LC ESI MS/MS

We designed a new liquid chromatography method with tandem mass spectrometry detection to concurrently measure cholic acid (CA) in plasma taken from blood samples. All chemicals, solvents, and analytic grade CA standards were obtained from Sigma Chemical. The internal standard (IS) was tolbutamide from Sigma Chemical.

5.7.4 Method validation of LC-ESI MS/MS bioanalytical method

This Cholic acid bio-analytical methodology was established according to US-FDA regulatory guidelines. The linearity of the plasma sample was measured using freshly prepared samples on three separate days. Y= mX+c, where m is the slope, was used to calculate the regression value. A method has been developed for the validation assay of a single cholic acid mix with plasma using **Tolbutamide** as an internal standard (IS). The freeze-thaw, autosampler, short-term, and long-term stability of LQC, MQC, and HQC samples were all measured. The matrix effect determined the lowest suppression of cholic acid ionization from plasma, whereas sample recovery determined the maximum extraction of cholic acid from plasma.

5.7.5 Calibration and quality control standard preparation for LC-ESI MS/MS

To prepare the internal standard, previously prepared 1mg/ml stock solutions of cholic acid and internal standard were diluted with methanol: water 50: 50 (v/v). 500ul of cholic acid was put into 500 ul blank plasma to create calibration standard concentrations 78.125, 156.25, 312.50, 625.00, 1250.00, 2500.00, and 5000.00, with LLOQ 78.125 ng/ml, LQC 234.375 ng/ml, MQC 1875.00 ng/ml, and HQC 3750.00 ng/ml.

5.7.6 Method validation for LC-ESI MS/MS

The methodology was validated using QC samples from the calibration curve at four concentration levels (78.125, 156.25, 312.50, 625.00, 1250.00, 2500.00, 5000.00 ng/ml). This technique was done four times over four days to measure the intraassay accuracy and precision. The percent relative error (RE) [% (measured - theoretical)/theoretical concentration] and relative standard deviation (RSD) [% RSD = % standard deviation/mean] were used to calculate accuracy and precision. The sample preparation recovery was evaluated by comparing the mean detector response of obtained QC samples spiked at low, medium, and high concentrations of LLOQ 78.125 ng/ml, LQC 234.375 ng/ml, MQC 1875.00 ng/ml, and HQC 3750.00 ng/ml in four replicates to that of extracted plasma blanks

spiked at equal concentrations. The matrix effect was estimated by comparing the recovered plasma residue to the clean solution. This bioanalytical method is highly selective, sensitive, and specific, with a low matrix effect and high recovery.

Analytical method development of Cholic Acid by LC-ESI-MS/MS

A method has been developed for validation assay of **Cholic Acid_**in plasma using **Tolbutamide** as an internal standard (IS).

OBJECTIVES:

To describe the development of proposed method of analysis of **Cholic Acid** from plasma.

CHEMICALS:

MeOH	HPLC Grade
Water	HPLC Grade (From Milli Q water purification system)
Ammonia Solution	AR Grade
Isopropyl Alcohol	AR Grade

CHROMATOGRAPHIC PARAMETERS:

Component Name	Triple Quadrupole LC/MS/MS Mass Spectrometer
Component ID	API 4000
Manufacturer	AB Sciex Instruments
Assay	1005760
Serial Number	V21410711
Column	Phenomenex Kinetex 5µ C18 100A 50*3mm
Mobile Phase	A: Milli Q water & 5mM Ammonium acetate with Ammonia solution (pH-9.92)
	B : 0.1% Ammonia solution in MeOH

SHIMADZU LC METHOD PROPERTIES :

Shimadzu LC system Equilibration time	0.00 min
Shimadzu LC system Injection Volume	10.00 ul

Shimadzu LC Method Parameters

Pumps

======

Pump A Model	LC-20AD
Pump B Model	LC-20AD
Pumping Mode	Binary Flow
Total Flow	0.5000 mL/min.

Auto sampler

==========

Model	SIL-20AC
Rinsing Volume	200 µL
Needle Stroke	52 mm.
Rinsing Speed	35 µL/sec.
Sampling Speed	15.0 µL/sec.
Purge Time	25.0 min.
Rinse Dip Time	0 sec.
Rinse Mode	Before and after aspiration
Cooler Enabled	Yes
Cooler Temperature	15 deg. C
Control Vial Needle Stroke	52 mm

Oven

====

Model	CTO-20AC
Temperature Control	Disabled

System Controller

==========

Model	CBM-20A
Power	On
Event 1	Off
Event 2	Off
Event 3	Off
Event 4	Off

QUANTITATION INFORMATION OF MASS SPECTROMETRY:

Scan Type	MRM (MRM)
Scheduled MRM	No
Polarity	Negative
Scan Mode	N/A
Ion Source	Turbo Spray
Resolution Q1	Unit
Resolution Q3	Unit
Intensity Thres:	0.00 cps
Settling Time	0.0000 msec
MR Pause	5.0070 msec
MCA	No
Step Size	0.00 Da

Cholic Acid	**Q1 Mass (Da)**	**Q3 Mass (Da)**	**Dwell(msec)**	**Parameter**	**Start**
	409.2	409.2	100.00	DP	-50.0
				CE	-50.0
				CXP	-15.0
Tolbutamide (IS)	**Q1 Mass (Da)**	**Q3 Mass (Da)**	**Dwell(msec)**	**Parameter**	**Start**
	268.7	169.8	100.00	DP	-45.0
				CE	-28.0
				CXP	-15.0

Parameters

==========

CUR	35.00
TEM	400.00
GS1	40.00
GS2	40.00
ihe	**ON**
IS	-4500.00
CAD	8.00
EP	-10.00

Internal Standard (IS):

==========

Drug Name	Concentration
Tolbutamide	1 µg/ml

5.7.7. Earthworm protein extraction and purification

Earthworms fully developed and between 15 and 20 cm long were chosen to prepare the crude extract. 500 g of adult earthworms salting in and salting out process both types of extract and pallet mix together then centrifuge (12,000x g) for 15 minutes at 4 °C. Then the homogenized autolyzed for 4 hours at 50°C in a 50 mM phosphate buffer solution with a pH of 6.0 and 0.2 percent sodium azide as a bacteriostatic. A 40 ml chloroform and methanol was used to homogenize 10 grams of homogenate, which was then kept at 4 °C for 2 hours. The homogenate received 50 ml of distilled water after 2 hr [2]. The mixture was then centrifuged for 10 minutes at 2460×g. The result was three distinct layers. It was pipetted off

the top water/methanol layer and completely evaporated on a rotavapor. Until use, an opalescent fluid with a pH of 7 was produced, freeze-dried, and stored at 4 °C. The Lowry protein assay determined the total protein content (Lowry). Dialysis membrane was performed after the freeze-dried earthworm extract in 50 mM phosphate buffer at a pH of 6.0 in 50 ml. The protein concentration passed through membrane dialysis was calculated. The gel filtration chromatography used Sephadex G 75 (GE Healthcare). Sephadex-G 75 beads were soaked in 50 mM phosphate buffer pH 6.0 for 48 h with slow stirring to avoid air entrapment in the matrix. The fully swelled beads were loaded on a polypropylene column with dimensions 30 × 2 cm. Sephadex-G75 beads were packed under gravity with a repeated flow of phosphate buffer to avoid air entrapment and cracking of the packed bed. The column was packed for a 15 × 2 cm dimension and preserved for 24 h at 10◦C to achieve compact packing. Then, 10 ml crude extract was loaded in the Sephadex-G 75 column and eluted with an excess of 50 mM phosphate buffer, pH 6.0, with an elution rate of 2 ml/min.

Then, after gel filtration (Sephadex G 75), MP was taken in 20 ml of a 20 mM phosphate buffer (pH 7.4), and the Amicon Pro Purification System, 50 kDa protein was purified. Fractions were collected, then visualized on SDS-PAGE pooled, and subjected to further characterization for future analysis[23].

5.7.8 protein quantification:

Here,the protein quantification by lowry and bradford assay. First, we remove the body parts from the earthworm. Then, we isolate its digestive organs. Then, make it like a paste. Then the pallets are collected from the salting-out process, and the proteins are collected from it by 50 kDa MWCO, where all the proteins below 50 kDa MWCO are taken. However, it can be said that the therapeutic efficacy of small proteins is much higher here. These proteins are dialyzed, and their protein collection is freeze-dried and taken in powder form. We do the quantification of each step by protein assay. Later, they were subjected to fast protein purification (FPLC) and their quantification [24].

5.7.9 Protein purification procedure

5.7.9.1 Salt purification:

The crude protein we used here was purified using a three-step chromatography method. We use the ammonium sulfate salt out process to perform a 1st step purification of the protein: ion exchange chromatography (DEAE cellulose) and size exclusion Chromatography (Sephadex G 200). The earthworm autolysate was filtered and centrifuged (12,000×g) for 30 min at 4 ◦C and precipitated with ammonium sulfate in the cold [25]. Degradation was suspended in 50 mM phosphate buffer pH 6.0, followed by dialysis. Dialysis Membrane (Hi-Media, Mumbai) Molecular weight cut-off (MWCO) of 14 kDa was pre-treated at 80 ◦C for 30 min with acid (1N HCl) and alkali (1N NaOH) in distilled water and preserved in 20% (v/v) ethyl alcohol. Then again, 3Kda Low molecular weight proteins were collected by running MWCO—15-ml Amicon centrifugal filter units 3 kDa MWCO (Millipore Sigma, catalog number: UFC800418) [26]. Protein samples were dialyzed against 50 mM phosphate buffer pH 6.0 for 2 h. The dialyzed sample is pooled and collected in a sterile vial for further study. In vitro studies of these proteins in our previous experiment showed that primary hepatocytes activate the regeneration process. Quantification of protein was done by the Lowry method. After each purification step, the fractions containing potential proteins were collected and analyzed using SDS-PAGE.

5.7.9.2 Size exclusion chromatography

Size exclusion chromatography: we use Sephadex G 50 (GE Healthcare). Sephadex-G 50 beads were soaked in 50 mM phosphate buffer pH 6.0 for 48 h to allow for a gradual size increase. After the beads were fully swollen, they were loaded onto a polypropylene column with a dimension of 30×2 cm. Sephadex-G50 beads were packed by gravity with repeated flows of phosphate buffer to avoid air entrapment and cracking of the packed bed. A 15x2 cm column was packed and stored at 10 ◦C for 24 h to achieve compact packing. 10 mL of fractin protein was placed on a Sephadex-G50 column and eluted with an additional 50 mM

phosphate buffer, pH 6.0. 2 ml/min [27]. A total of 30 different fractions were collected, and each Fraction was analyzed for protein content and regeneration activity. The fraction with maximum activity visualized SDS-PAGE was pooled and further characterized.

5.7.9.3 Fast protein liquid chromatography (FPLC) protein purification for chromatography:

Ion exchange chromatography used Diethylaminoethyl-cellulose (DEAE-cellulose) resin (GE CYTIVA) on the FPLC machine. It was activated by pre-treatment with acid (1N HCl) and alkali (1N NaOH) and finally rinsed with an excess of distilled water. Further, the charged resin was incubated at room temperature for 2 hours in 50 mM phosphate buffer pH 6.0. buffer (50 mM, phosphate buffer, pH 6.0). The column was washed with wash buffer (50 mM phosphate buffer, pH 6.0) to remove unbound protein from the column [28]. A total of 15 fractions were collected at 2ml/min, and each fraction Activity was measured and evaluated for protein content. Fractions exhibiting maximum activity were pooled and Subject to size exclusion chromatography. Purified protein was evaluated on a 12% polyacrylamide gel on SDS-PAGE with standard protein markers.

5.7.9.4 In-gel digestion or trypsin digestion:

Rinse the entire slab of a one- or two-dimensional gel with water for a few hours, put a plastic tray with the gel onto a lightbox, and excise bands (spots) of interest with a clean scalpel. Cut excised bands (1d gel band). Transfer gel pieces into a microcentrifuge tube and spin them on a bench-top microcentrifuge [30]. If rapid identification of Coomassie-stained bands is intended, skip (reduction/alkylation process). Add 100 ml of 100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) and incubate with occasional vortex for 30 min, depending on the staining intensity. Add 500 ml of neat acetonitrile and incubate at room temperature with occasional vortex until gel pieces become white and shrink, and then remove acetonitrile. Although the bulk of Coomassie staining should be removed, it is unnecessary to destain the gel pieces completely. Add enough trypsin buffer to cover the dry

gel pieces (typically, 50 ml or more, depending on the volume of a gel matrix) and leave it in an ice bucket or a fridge. After 30 min, check if all solution was absorbed and add more trypsin buffer, if necessary. Gel pieces should be completely covered with trypsin buffer [31]. Leave gel pieces for another 90 min to saturate them with trypsin, and then add 10–20 ml of ammonium bicarbonate buffer to cover the gel pieces and keep them wet during enzymatic cleavage. Place tubes with gel pieces into an air circulation thermostat and incubate samples overnight at 37℃ for analyses performed at the limit of instrument sensitivity, which requires maximal peptide recovery. Otherwise (typically, for the rapid identification of Coomassie stainable spots (bands) by MALDI mass fingerprinting), the acceptable digestion yield—exceeding, on average, 75% of the yield of overnight cleavage—can be achieved in 30 min at 55℃ If the protein digest needs to be further analysed by MALDI TOF MS, proceed to next step; otherwise, proceed to Step 10 directly [31][32]. Chill tubes to room temperature, spin down gel pieces using a microcentrifuge and withdraw 1–1.5 ml aliquots of the supernatant directly from the digest without further extracting the gel pieces. A typical volume of the digestion buffer is approximately 50 ml, leaving ample peptide material for the subsequent MS/MS analysis if required. Add 100 ml of extraction buffer (1:2 (vol/vol) 5% formic acid/acetonitrile) to each tube and incubate for 15 min at 37 ℃ in a shaker. For samples with a much larger (or smaller) volume of gel matrix, add the extraction buffer such that the approximate ratio of 1:2 between the digest and extraction volumes is achieved. For further LC-MS/MS analysis, add 10–20 ml of 0.1% (vol/vol) trifluoroacetic acid into the tube, vortex, and/or incubate the tube for 2–5 min in the sonication bath and centrifuge for 15 min at 6.7g (10,000 r.p.m.) at the bench-top centrifuge and withdraw the appropriate aliquot for further analysis. Dry down the rest in a vacuum centrifuge and store at 20 ℃ as requard [33].

5.8 Result

5.8.1. Acute oral toxicity (single dose) test

During the acute toxicity testing phase, careful observation of animals was conducted to identify any indications or manifestations of toxicity. These observations were made at various time intervals, namely every 15, 30, 60, 120, 180, and 240 minutes and once daily for a period of 14 days. In the acute toxicity investigation, no clinical parameters indicating toxicity were detected in either the vehicle control or the treatment groups. Throughout the investigation period, the animals exhibited good health. Additionally, no apparent alterations in behaviour or unusual variations in water and food consumption were noted among the animals. However, hyperactivity was noticed shortly after the therapy, although this effect subsided within a 24-hour period. Furthermore, no mortality associated with therapy was seen following the administration of MP at a single dose of 2000 mg/kg b.w. However, female rats treated with MP showed a 3.5 % ($p < 0.05$) increase in body weight and a 34.21 % ($p < 0.05$) increase in the relative weight of the heart when compared to the vehicle group **(Table 1)**. Also, a 12 % ($p < 0.01$) increase in the relative weight of the kidney of female rats was observed compared to the control group (Please arrange the table in a more informative way and show 12 % increase in the table). No macroscopic changes were observed in the organs analysed. Although the excretion of black faeces was noticed promptly following the administration of a dose of 2000 mg/kg, but this disappeared within a 24-hour period. On the other hand, the result of body weight in the acute toxicity test is shown in Table 2. The body weight of rat showed significant variations in the mean of all groups after 14 days tested compared to the start time (0 h) of the acute toxicity study ($p < 0.05$). However, the body weight of rat at 24, 48, 72 h, and 7 days ($p > 0.05$) after MP administration showed a statistically no significant difference compared to the time of initial treatment. Besides, the body weight gain showed no significant difference (p value >0.05) between the control and

treated groups. This result showed that MP did not affect the increase in body weight of rat. The cause may be that the rat grew and gained weight over time during the test.

Table 1. Effect of single acute oral administration of crude extract of *Metaphire Posthuma* on body weight and relative organ weight of Rat at the end of 14 days.

Dose	Body weight (g)		Relative organ weight (%)			
	Initial Body Weight Day 0	Final Body Weight Day14	Lungs	Liver	Kidney	Heart
Vehicle	165 ±0.4	170±0.2	0.72±0.2	4.84±0.3	0.86±0.5	0.38±0.4
2000 mg/kg (Male)	165±03	174±0.3*	0.72±0.02	4.87±0.4	0.91±0.4	0.41±0.5
2000 mg/kg (Female)	165±03	176±0.3*	0.74±0.02	4.93±0.4	0.97±0.3	0.51±0.6

Table 2. *Body weight of male and female albino Wister rat for acute toxicity investigation 14 days:*

Dose	*0h*	*8h*	*16 h*	*24 h*	*48 h*	*72 h*	*7 days*	*14 days*
Vehicle	*165.03± 2.4*	*165.05±3.5* *0.012%*	165.09±1.4 *0.036%*	166.02±2.7 *0.59%*	166.08±1.5 *0.63%*	166.09± 0.6 *0.64%*	167.05± 3.9 *1.24%*	170.05± 2.3 *3.04%*
2000 mg/kg Male	165.05± 1.5	165.09±2.2 0.024%	166.04±3.5 0.59%	166.09±2.5 0.63%	167.05±1.5 1.29%	169.27± 1.5 2.5%	170.15± 2.5 3.08%	174.05± 1.5 5.45%
2000 mg/kg Female	165.05± 1.5	165.05±1.5 0.006%	166.16±2.5 0.67% ↑	166.89±1.5 1.11% ↑	168.39±1.5 2.02% ↑	170.49± 2.5 3.29% ↑	171.69± 1.5 4.02% ↑	176.03± 1.5 6.65% ↑

5.8.2. Subacute toxicity (repeated doses)

5.8.2.1. Body weight

Changes in the weights of both the treated and control groups were recorded (as shown in Table 3). A steady (significant or non-significant don't use steady) increase in the weights of all mice groups was noticed throughout the sub-acute study. From day 14 onwards, a notable weight gain was observed in all treatment and control groups compared to their initial weights on day 1. By day 28, the body weights of three treated groups and one control group had increased by 8.05%–10.55% relative to their weights on day 1. The findings suggest that the treatment did not result in any statistically significant changes.

Table 3. Body weight male rat sub-acute toxicity investigation 28 days

Dose	Day 1	Day 7	Day 14	Day 21	Day 28
500 mg/kg	160.15±1.3	164.03±2.6 2.42% ↑	168.44±1.9 5.17% ↑	174.33±2.1 8.85% ↑	176.12±2.4 9.97% ↑
1000 mg/kg	150.04±2.1	154.12±1.7 2.71% ↑	156.88±1.4 4.55% ↑	160.88±1.1 7.22% ↑	165.88±1.2 10.55% ↑
1500 mg/kg	155.34±1.1	157.22±2.1 1.21% ↑	160.85±1.8 3.54% ↑	164.85±2.1 6.12% ↑	167.85±2.4 8.05% ↑

5.8.2.2. Behavioral and physical parameters

Rats that received varying doses of MP (500, 1000, or 1500 mg/kg/day) for a period of 28 days did not exhibit any alterations in behavior, nor did they display any indications or manifestations of toxicity, such as alterations in skin pigmentation, ocular abnormalities, respiratory impairments, gastrointestinal disturbances, or changes in the central and autonomic nervous systems. No fatalities of the rats were detected over the assessed time frame. Furthermore, there was no observed variance in the consumption of feed and water among the animals. Additionally, no discernible alterations were detected in the patterns of urine and faecal excretion during the specified period for the low and medium dosages. In addition, the study findings indicate that there was no statistically significant difference in food consumption and water intake between the control group and the treatment group for the whole duration of the study. Although in a sub-acute examination, black stool excretion was observed following 20 days of MP therapy at a dosage of 1500 mg/kg/day body weight in comparison with the control group.

5.8.2.3. Haematological and biochemical analyses

The findings indicated a substantial decrease in haematocrit values of the rats across all three dosage levels of MP. The decreases were as follows: 4.1 % ($p < 0.01$) for the 500 mg/kg dosage, 2 % ($p < 0.05$) for the 1000 mg/kg dose, and 23 % ($p < 0.01$) for the 1500 mg/kg dose in particular. A significant decrease in total lymphocyte count of 8 % ($p < 0.05$) was seen in rats administered with a dosage of 500 mg/kg of MP, as compared to the control group. The quantity of monocytes in the whole of the blood of animals subjected to doses of 500, and 1000 mg/kg MP exhibited a statistically significant reduction ($p < 0.05$) of 19 %, and 9 %while an increment of 9.5 % was observed for the 1500 mg/kg treated group, in comparison to the control group. A reduction in AST levels of 19 %, 25 % and 36 % ($p < 0.05$) was seen in the 500, 1000 and 1500 mg/kg MP treated groups. Furthermore, the ALP levels also found to be reduced by 12 %, 13.92 %, 15 % in 500, 1000 and 1500 mg/kg respectively. The remaining haematological parameters exhibited no changes, as indicated in Table 4.

Table 4. Effect of sub-acute oral administration crude extract of *Metaphire posthuma* on haematological and biochemical parameters in rats at the end of 28 days.

Parameters	**Vehicle**	**Crude Extract of *Metaphire Posthuma* (in mg/kg/day)**			**Reference value**
		500	1000	1500	
Glucose (mg/dl)	121±4	117±6	124±3	123±8	**76-176**
Serum sodium(mmol/l)	147±6	141±4	144±5	**125±7***	**142-151**
Blood pressure NIBP (mmhg)	134±2	141±7	146±8	**118±5**	**125-145**
Haemoglobin (g/dL)	14.8±0.5	14.4±0.9	14.8±0.6	15.3±0.5	**11-19.2**
Red blood cell(10^6/µl)	8.7±1.2	7.99±0.7	8.2±0.9	8.5±2.3	**7.29-9.65**
Platelets(10^3/µl)	1034±10	994±6	1039±6	1078±12	**638-1177**
Red blood cell (10^6/µl)	8.11±0.3	8.01±0.8	8.11±0.5	8.21±0.9	**7.27-9.65**

White blood cell (10^3/µl)	5.11±0.4	4.73±0.3	5.08±0.7	5.12±0.7	**1.96-8.25**
Neutrophil (%)	13.34±0.5	12.12±0.5	16±0.9	15±0.8*	**6.2-26.7**
Lymphocytes (%)	85.6±0.9	78±0.7	81±0.7	87.4±0.2	**66.6-90.3**
Monocytes (%)	2.1±0.7	1.7±0.6	1.9±0.2	2.3±0.4	**0.8-3.8**
Eosinophils (%)	1.7±0.4	1.9±0.7	2.3±0.3	2.2±0.8*	**0.2-3.5**
Basophils (%)	0.04±0.004	0.5±0.008	0.03±0.007	0.03±0.009	**0-0.08**
Total protein(g/dl)	6.9±1.3	7±0.9	7.1±1.2	7.1±0.5	**5.6-7.5**
Total albumin(g/dl)	3.9±0.6	4.1±0.6	4.2±0.3	4.1±1.2	**3.4-4.8**
Globulin (g/dl)	2.1±0.2	1.8±0.4	1.9±0.8	2.3±0.6	**1.5-2.5**
A/G ratio	2.1±0.8	1.9±1.03	1.89±0.7	2±0.8	**1.58-2.67**
Alkaline phosphate (U/L)	128±6	138±9	136±7	133±10**	**62-240**
Aspartate aminotransferase (U/L)	**32±3**	36±2.7	29±4.1	37±6	**35-40**
Alanine aminotransferase (U/L)	35±2.1	**30±3.1**	**27±2.9**	**34±3.8**	**35-45**
Urea (mg/dl)	17.9±0.9	21.1±1.6	20.6±1.8	19.3±2.1	**12.4-25**
Creatinine kinase (U/L)	560±20	480±30*	560±10	597±21**	**163-1045**
Creatinine mg/dl	0.39±0.18	0.42±0.11	0.57±0.19	0.61±0.2*	**0.4–0.8 mg/dl**
Direct bilirubin(mg/dl)	0.04±0.002	0.01±0.004	0.03±0.006	0.02±0.007	**0.03-0.06**
Indirect bilirubin(mg/dl)	0.09±0.006	0.1±0.002	0.07±0.005	0.08±0.004	**0.03-0.15**
Total bilirubin(mg/dl)	0.14±0.02	0.09±0.06	0.11±0.05	0.12±0.08	**0.05-0.18**
Triglycerides (mg/dl)	45±2.4	42±4.1	44±2.9	40±3.1*	**14–46**

All blood parameter, Blood pressure NIBP (mm hg), and Reference values for Wistar rats were given. Here all values are representing Mean ± SD. (n = 6/group). One-way ANOVA, followed by the Tukey test. *$p < 0.05$; **$p < 0.01$ and versus Vehicle.

5.8.2.4. Relative organ weight and macroscopic and histopathological analyses

The relative weight of the heart, lung, liver, and kidneys did not change after 28 days of therapy with crude *Metaphire posthuma* extract at 500, 1000, and 1500 mg/kg/day, p. o. Fig.

4 shows histological sections of the heart, lung, liver, and kidney of male rats from the control and treatment groups after 28 days of oral gavage with crude *M. posthuma* extract at 500, 1000, and 1500 mg/kg/day, p.o. Macroscopy demonstrated that the organs of rats given three doses of *M. posthuma* crude extract had intact topography. The histopathological findings indicated that a treatment dose of 1500 mg/kg/day body weight resulted in the development of a very mild form of interstitial nephritis, in comparison with the control group which exhibited little inflammation in the interstitial tissue and renal tubules, along with the infiltration of inflammatory cells. Still, no substantial pathological alterations were seen throughout the time frame of 28 days, while administering treatment dosages of 500, 1000, and 1500 mg/kg/day body weight.

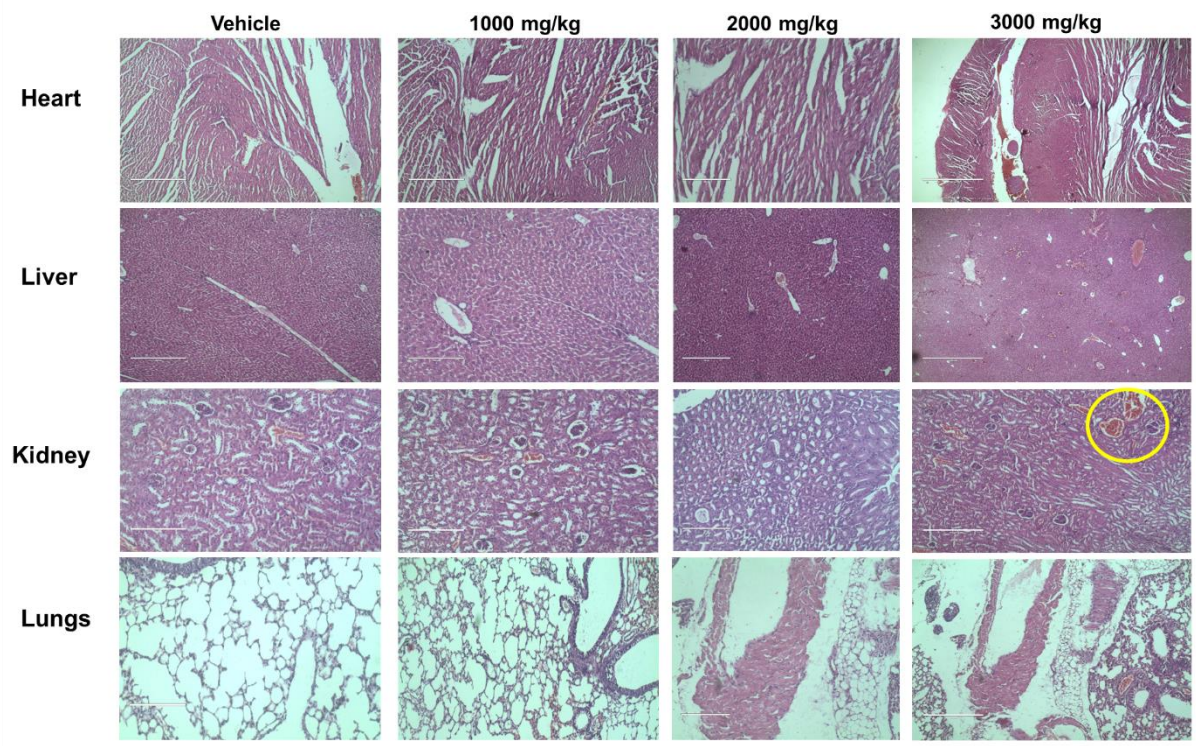

Figure 1. Histopathological examination of different tissues (heart, liver, kidney, lungs) of treated rats with MP and with vehicle. The crude extract of *Metaphire posthuma* doses 500, 1000 and 1500 mg/kg/day, p.o., was administered for 28 days.

5.8.3. In-vitro test

5.8.3.1. In-vitro primary hepatocyte trypan blue exclusion test in EtOH induce hepatotoxicity:

Administration of ethyl alcohol (EtOH) decreased the viability in hepatocytes group II with 47.69 % after day 14. Whereas group I was kept as control with the percentage of cell viability of hepatocyte was 87.69%. But in other treatment groups (III, IV, V and VI) the percentage of cell viability increased significantly (57.89 % 64.13 %, 74.3 %, and 81.31 %) as shown in (Table 1).

Table 5. Preventive effect of EtOH, GM, MP and GM+MP on viability of isolated rat hepatocytes against EtOH-induced (4 g/kg p.o.×14 days) toxicity

Parameter	Group I	Group II alcohol only	Group III	Group IV	Group V	Group VI
Percentage viability (Trypan blue exclusion test)	87.69±2.51	47.69±3.27	57.89±3.21	64.13±3.35	74.31±2.25	81.31±3.215

All values are mean ±S.D. of six animals. Group II compared with group I: P<0.001 in all values. Group III compared with group II: P<0.001 in all values.

5.8.4. AST/ALT ratio, Albumin, Total Bilirubin, Cholesterol, and Triacylglycerides EtOH induced hepatotoxicity

The results of group II (2.8±0.37) disease control increased significantly ($p < 0.05$) compared to group I but the treatment groups III, IV,V,VI significantly ($p < 0.05$) lowered AST /ALT ratio respectively (2.2±0.09, 1.9±0.27, 1.5±0.22, 1.4±0.19) in (Fig.2A). The percentage decrease in AST/ALT ratio of group III, IV, V and VI are 32.98%, 51.05%, 76.62%, 80.70% respectively when compared to group II. Group I to Group II showed a significant decrease ($p<0.05$) in albumin with values of 3.5±0.067 g/dL and 2.14± 0.05 g/dL respectively. Furthermore, it was found that among the animals treated in groups IV,V, and VI, a trend towards normalization of albumin values (2.5, 2.6, and 3.08 g/dl respectively) which was very similar to that of group I were observed in (Fig 2B). Percentage of increase of albumin for group III, IV, V and VI were 23.03 %, 27.92 %, 37.82 %, 67.78 % respectively to that of group II in (Fig 2B).The total bilirubin level in group II increased significantly from group I

which indicates the increase value (3.76±0.51). On the other hand, all the treatment groups except group III significantly decreased this total bilirubin. Their levels are shown in (Fig 2C) group IV, V and VI respectively (2.63±0.49,2.38±0.44 and 2.1±0.8 mg/dl) reduced. Total Bilirubin percentage of inhibition was found to be 16.04 %, 33.48 %, 41.35%,49.37 % than group II in (Fig. 2C). In the case of cholesterol, it was observed that group II (25.16 mg /dl) significantly ($p < 0.05$) reduced the cholesterol level from group I (55.3 mg /dl). While in the treatment groups especially (III, IV, V, VI) the cholesterol level was found to gain normalization like the control group I. Further to mention, the results of treatment group V and VI (37.3 mg /dl, and 38.3 mg /dl) changed significantly ($p < 0.05$) from group II (25.1 mg /dl) (Fig. 2D). The changes in cholesterol percentage of increase is depicted in (Fig 2D). The percentage of increase of cholesterol for group III, IV, V and VI were 4.41 %, 11.60 %, 40.33 %, 43.64 % respectively when compared with group II. According to the results (Fig 2D), suggests that in group II significantly ($p < 0.05$) increased triglyceride concentration (101.33 mg/dL) compared to all experimental groups. However a significant change was seen in group V and VI among remaining four groups compared to group II . Where the percentage of inhibition of TG for group III, IV, V and VI was found to be 15.78 %, 28.57 %, 30.07 %, 39.84 % respectively when compared with group II

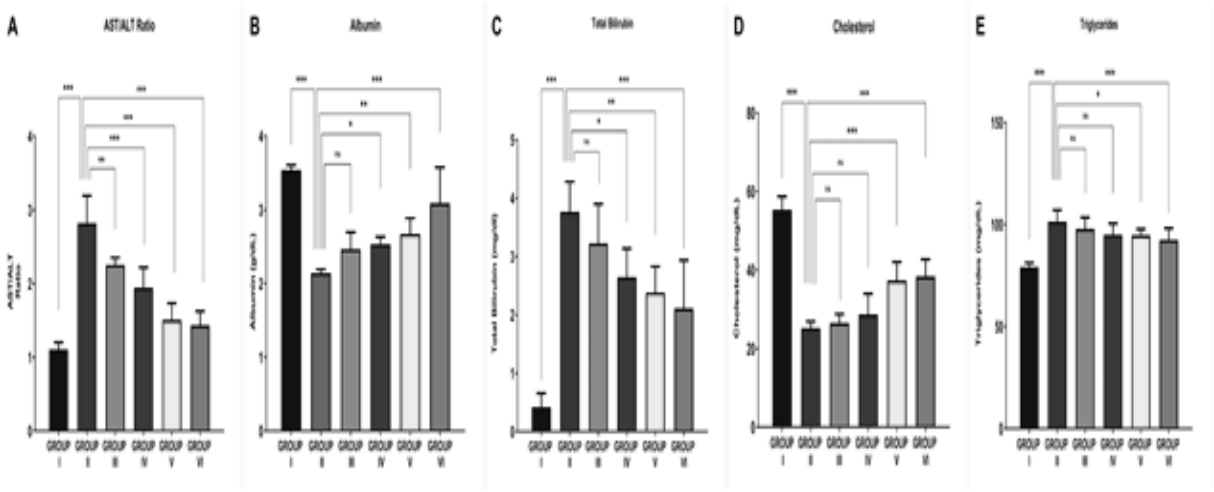

Figure 2. Effect of EtOH, GM, MP & GM+MP on Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ratio (AST/ALT) in (Fig 2A), (Fig B) albumin, (Fig 2 C) total bilirubin, (Fig 2 D) cholesterol and (Fig 2 E) triglycerides in Ethanol 4g/kg bd.wt (EtOH)-induced in rats. Data are presented as mean ± SD (n=6) and analysed one-way ANOVA test followed by Tukey's post hoc test. ***, **, *** and** **ns(not significant)** Significantly different from the normal control group I, EtOH-intoxicated group(II), and GM-treated group (III),MP-treated group (IV),GM+MP treater group (V) & (VI) respectively, at (*$p < 0.05$, **$P < 0.01$ and ***$p < 0.001$). NC: normal control group (I).

5.8.5. Anti-oxidant, inflammatory and metabolite activity EtOH induced hepatotoxicity:

Catalase (CAT) activity results indicated that group II results showed a significant decrease ($p<0.05$) compared to group I value (11.33 nmol/min/mg protein) (Fig 3A). Similarly, in the treatment groups, the value of CAT increased significantly in groups V and VI with values (14.3333 and 14.6667 nmol/min/mg protein) respecively. (Fig 3A) demonstrates the percentage increase in CAT values among the treatment groups III, IV, V and VI with13.46 %, 25 %, 28.84 %, and 38.46 % respectively when compared to group II. Also, GSH activity, of the liver, in group II (1.26333 μM/mg/protein) was found which is significantly decreased ($p < 0.05$) than group I (3.41833 μM/mg/protein) (Fig 3B). Moreover, according to the results obtained, the treated groups had almost restored the same level of GSH as in group I. While, there was a significant increase in the percentage of GSH in all treatment groups III, IV, V and VI (29.23 %, 48.56 %, 59.55 %, 78.03 %) compared to group II. Similarly, the value of TNF-α, an inflammatory marker (Fig 3C), was found to be significantly increased in group II (118 pg/ml) from that of group I (23.16 pg/ml). Whereas, the treatment groups III, IV, V and VI showed significant reduction. The values of TNF-α in the treatment group III, IV, V and VI were 89.3, 88.3, 79.3 and 73.8 pg/ml respectively. Further to mention, (Fig 3C) shows the percentage of inhibition of TNF-α in treatment groups III, IV, V and VI (30.22 %, 31.28 %, 40.77 %, 46.57 %) compare to group II. Again, the level of another inflammatory marker IL-6 (Fig 3D) was significantly increased in group II, 295.83 pg/ml, from group I (73.6 pg/ml). In this case too, level of IL-6 was found to be significantly less in the treatment groups (III, IV, V and VI). The results of treatment groups (III, IV, V and VI) were 226.3, 218.3, 182.50 and 158.50 pg /ml respectively. Thus, indicating that the inflammation was greatly reduced. Also (Fig 3D) depicts the change in percentage of inhibition of IL-6 of treatment gropus III, IV, V and VI with 31.28 %, 34.88 %, 43.51 %, 61.81 % than group II. CYP2E1 is an enzyme that has a special role in metabolism and oxidative stress. The value of CYP2E1 in Group II (473.66 ng /ml) increased significantly (Fig 3E) from Group I (197.83 ng/ml). Also, the result of treatment group V and VI (362.5 and 308.5 ng/ml) has decreased significantly ($p < 0.05$). The percentage of inhibition in treatment groups III, IV, V and VI compared to group II were found to be 27.91 %,33.29 %,40.30 %,59.87 % respectively (Fig 3E).

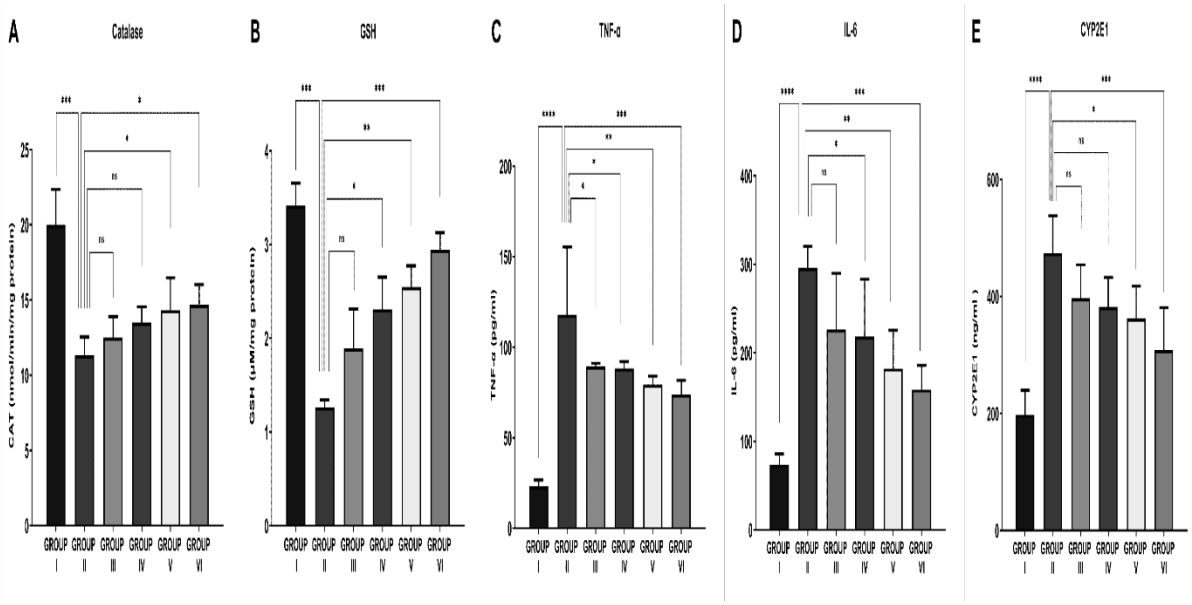

Figure 3. Effect of EtOH, GM, MP, and MP+GM in treated group (III,IV,V and VI) on (Fig 3A) catalase (CAT), (Fig 3B) glutathione (GSH), (Fig 3C) TNF-α, (Fig 3D) IL-6, and (Fig 3E) CYP-2E1 in Ethanol 4g/kg bd.wt (EtOH)-induced in rats as group II. Data are presented as mean ± SD (n=6) and analyzed by a one-way ANOVA test followed by Tukey's post hoc test. *, **,*** and $^{ns(not\ significant)}$ were Significantly different from the normal control group I, EtOH-intoxicated group II, and GM-treated group III, MP-treated group IV, GM+MP treater group V & VI respectively, at (*p < 0.05, **P < 0.01 and ***p < 0.001). NC: normal control group I.

5.8.6. Activities of liver function, oxidative, Antioxidant activity and inflammatory cytokine activity in MP and CCl_4 induce activity:

Hepatotoxicity was dramatically increased in ALT, AST, Total protein and bilirubin levels compared to group I and group II CCl_4 induce hepatotoxicity. Unlike the treatment group (III, IV,V), MP therapy significantly reduced the elevation of ALT, AST, and bilirubin levels (*p < 0.05, **P < 0.01 and ***p < 0.001). The activities of MDA and GSH were measured to evaluate how MP affects CCl4-induced oxidative stress in rats. One of the byproducts of lipid peroxidation is malondialdehyde (MDA), which is often used as a biomarker of oxidative damage to the liver. MDA levels were significantly higher in CCl_4-treated group (II) than in group (I), although MP administration at doses of 50, 100, and 200 mg/kg significantly

inhibited the ability of CCl_4 to cause an increase in MDA in the liver (*p < 0.05 , **p < 0.01 and ***p < 0.001). But for chronic toxicity, especially 12 week CCl_4 induces total protein level to 4.5 g/dl in group II. On the other hand, MP treatment results in 6.08, 6.15, 6.8 and 6.9 g. /dl. Pro-inflammatory cytokine levels, especially TNF-α, IL-6, and STAT-3 in group II increased significantly compared to group I (***p<0.001). On the other hand, MP treatment groups significantly increased respectively (*p<0.05, **p<0.01, and ***p<0.001). But in case of IL 4, the level of group I to group II was significantly increased ***P<0.001. On the other hand, the level of MP treatment group III did not change significantly from group II, but the level of IL4 decreased significantly in MP treatment group IV and V (**P<0.01 and ***P<0.001) in (Fig 4).

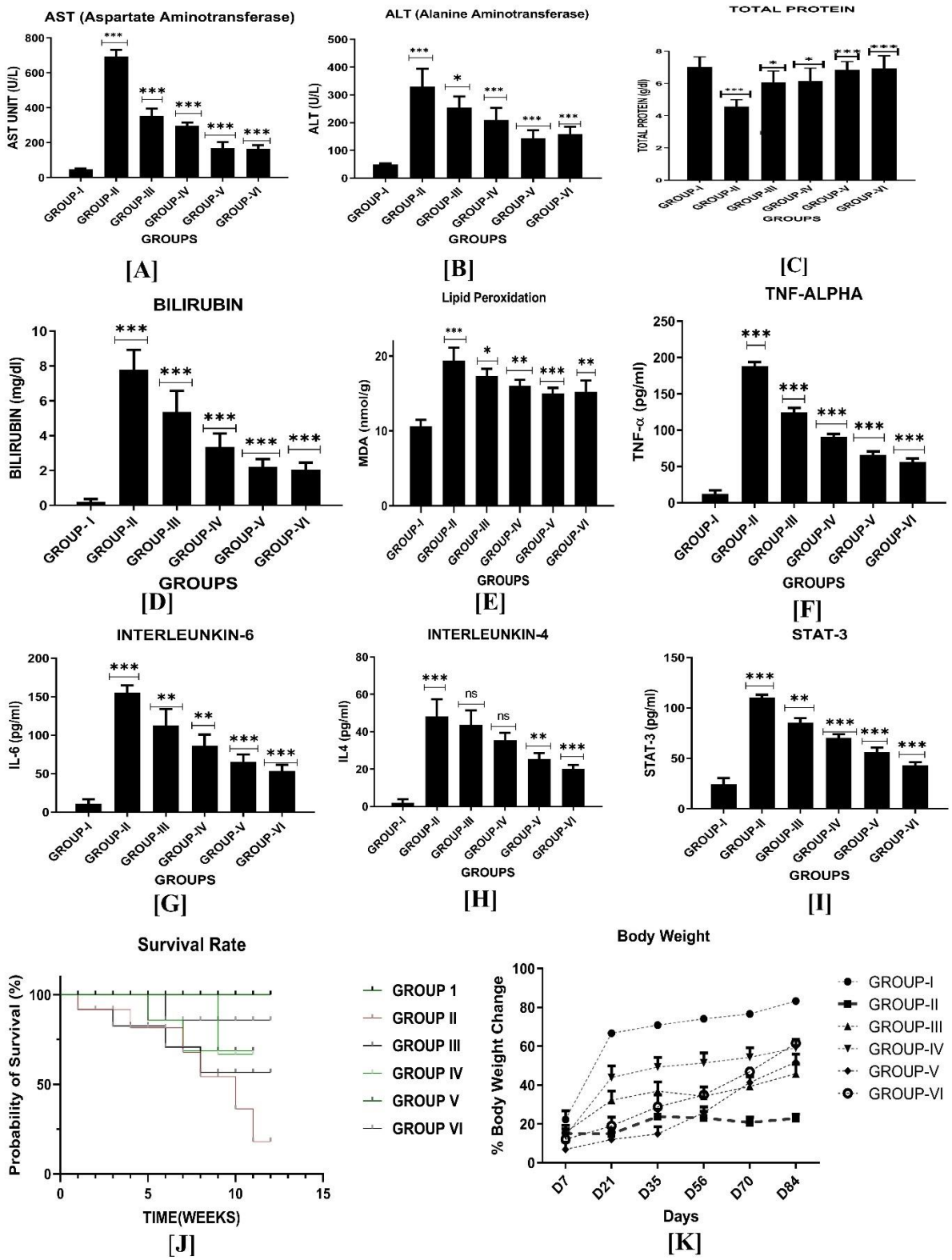

Figure 4. The MP showed in (Fig 4A,4B,4D &4E) AST, ALT, Bilirubin, and lipid peroxidation of MDA level has significantly *($p<0.05$), **($p<0.01$), ***($p<0.001$),****($p<0.0001$) changed. (Fig 4C),) Total protein levels show a change in group II than treatment groups. In ELISA test (Fig 4F,4G,4H and 4I), TNF-α, IL6, IL4, and STAT3 levels significantly changed in (Fig 4). Here also show (Fig 4J & 4K) survival rate and body weight changed significantly ($p<0.05$).

5.8.7. mRNA expressions of AKT1/α-SMA/ Bax /NRF2 in CCL4 hepatotoxicity:

In our experiment, CCl_4 induces disease control group (II) animals to express higher levels of the α-SMA, AKT1, and Bax genes for severe liver damage obtained in the gel-based PCR for qualitative investigation. The positive rise in α-SMA, AKT1, and Bax gene expression was shown in gel images to be higher in the disease control group (II) than in the test groups. Here, NRF2 expression in the treatment group showed increased antioxidant activity in (Fig 5).

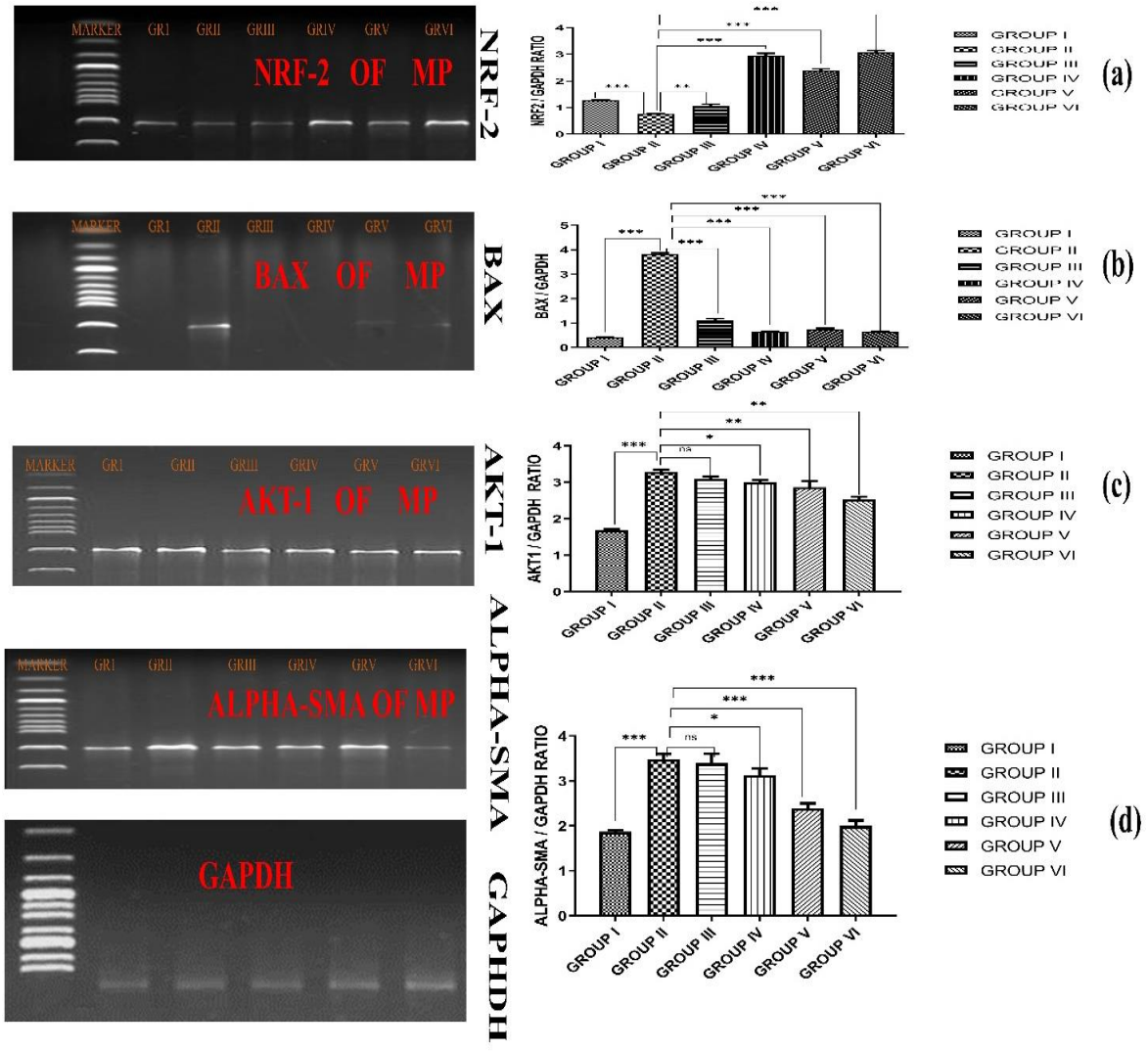

Figure 5. The mRNA expressions of NRF2, BAX, AKT1, α-SMA and the housekeeping gene GAPDH are shown qualitatively. Densitometry imageJ software analysis here shows that the ratio of BAX, AKT1, and α-SMA expression is increased (Fig 5). in (a),(b),(c), and (d) respectively. Here, the disease control group II showed an increase in cytokine or proinflammatory activity compared to the experimental group. Antioxidant activity increased in the treatment group (Fig 5a). Data are expressed as mean ± SEM, *p<0.05, **p<0.01,

***p<0.001 versus normal control group I and disease control group II and treatment group (III, IV, V, and VI) in (Fig 5).

5.8.8. Effect of body weight MP, GM, MP+GM, and EtOH induced hepatotoxicity:

An increase in BW (body weight) was observed in the MP + GM-treated groups, with values higher than those in group II. Group (II, III, IV, V and VI) percentage of body weight gain was (9.9%, 13.95%, 16.91%, 23.32% and 34%), respectively, these results were higher in treatment group in (Fig 6C). Compared with group I, EtOH group II had a change in body weight (0.91%). Which means body weight group II increased less than normal. Moreover, no mortality was observed in group II. Also, groups V, and VI significantly changed body weight from day 0 to day 14 of the study which was similar to group I as shown in (Fig 6A).

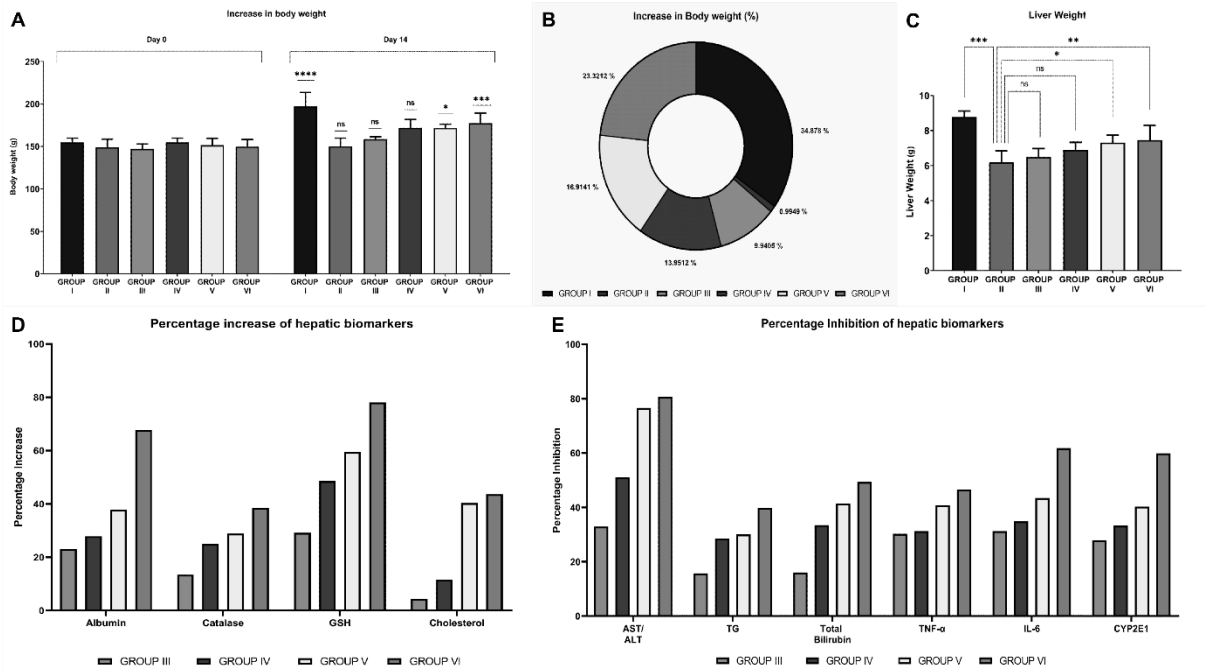

Figure 6. In (Fig 6A) Represent the body weight increase (day zero to day 14). Only Group (I), (IV) & (VI) has significantly show their changed body weight, p-value ****(p<0.0001), *(p<0.05) and ***(p<0.001). (Fig 6B) represents mean ± SD of liver weight group (I) vs (II) has significantly change p-value ***(p<0.0001). Group (II) vs (V) and (VI) significantly increase liver mass p-value *(p<0.05), **(p<0.01). Here, group (V) and group (VI) liver mass value increased, similar to group (I). (Fig 6B) Group (II,III, IV, V and VI) percentage of body weight gain was (9.9%, 13.95%, 16.91%, 23.32% and 34%), respectively, these results were higher in treatment group in (Fig 6C,6E) showed here the percentage inhibition in group (III, IV, V and VI) AST/ALT Ratio (32.98%, 51.05%,76.62%,80.70%), Cholesterol

(4.41%,11.60%,40.33%,43.64%),Triacylglycerol(15.78%,28.57%,30.07%,39.84%),CYP2E1(27.91%,33.29%,40.30%, 59.87%).

5.8.9. Survival rate & MP restored weight loss in CCl_4-induced rats:

The control group (I) had no recorded instances of animal mortality. However, within the second group (II), it was shown that 35% of the animal subjects experienced mortality due to chronic poisoning. The mortality rate in group III was 20%, whereas in group IV, it was 5%. No fatalities were recorded in either group (V) or group (VI), as shown in (Fig 4J). The weight of the control group (I) had nearly doubled (92.16%) at the end of the 12-week treatment period and was significantly higher ($P < 0.05$) than the disease control group (II), which increased by just 27.01%. MP demonstrated dose-dependent weight-gain management in rats (200 mg/kg), with a 51.75% rise in body weight in (Fig 4K).

5.8.10. Histopathology EtOH-induced hepatoxicity:

It has been noted that distinct alterations in the morphological and functional properties of hepatocytes take place following hepatic resection. The histopathological finding indicates EtOH Group (II) animals were (Fig 7B) increased in apoptosis, necrosis, and congestive hepatopathy, which supported the presence of inflammation and bile duct damage in the histological sections of the liver. In contrast to the histopathological study for the rats treated with MP revealed a decrease in the severity of the above-mentioned histological alterations.

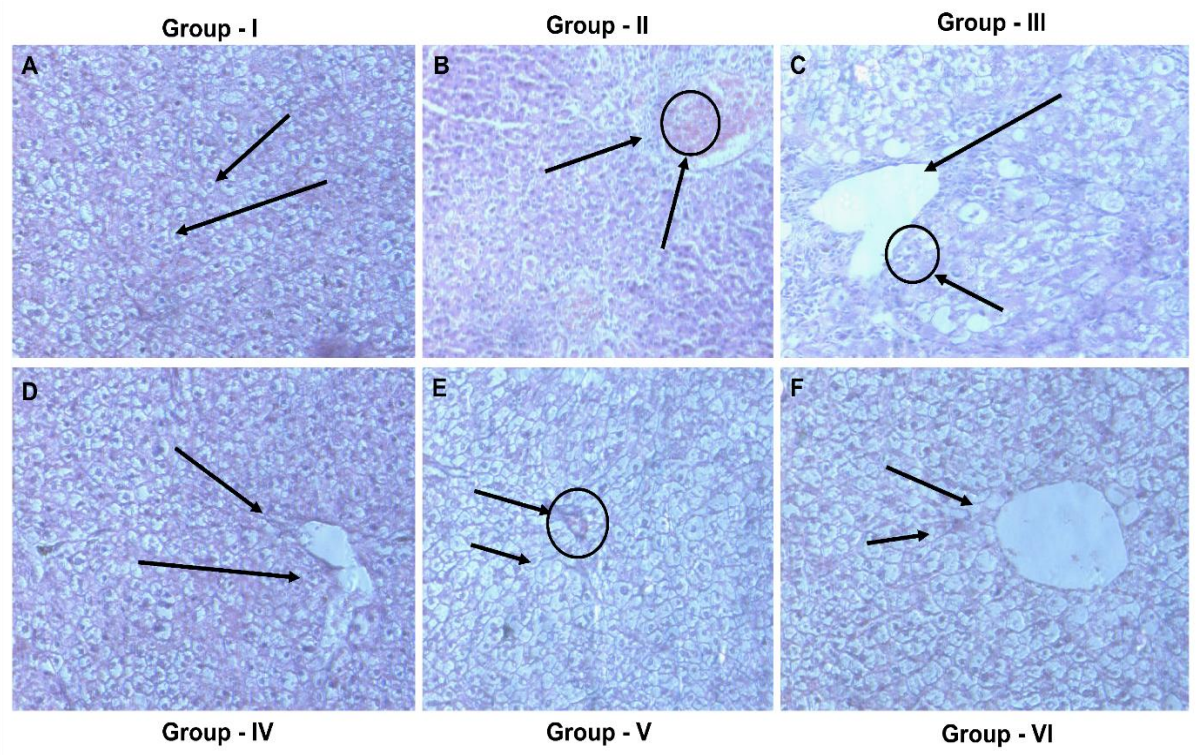

Figure 7. Histological patterns of liver. (Fig 7A) The control group is shown with normal liver histology. (Fig 7B) Group II has excessive damage of EtOH, inflammation (circle, arrow), fatty cells, damage hepatocytes, microvascular steatosis (circle), necrosis, shown (circle), bile duct damage, inflammation.In (Fig 7C) moderate hepatic cell damage and steatosis shown (circle) in group (III) animal. (Fig 7D) Hepatocyte damage decrease, fatty cell does not appear, small fatty change observes, few hepatic cell abnormalities observe, liver cellular architecture moderate change in group (IV). (Fig 7E) decrease in necrosis and microvascular steatosis, hepatocyte cell not abnormal shown (arrow sign) in group (v). (Fig 7F) liver cell normal structure, apoptosis, microvascular steatosis not appear seriously in group (VI) rat's liver.

5.8.11. Histopathology of CCl_4 induced hepatotoxicity

CCl_4 produced the rat liver fibrosis model to assess the preventive impact of MP against chronic liver fibrosis. The biochemical and histological investigations proved that the rats given CCL_4 experienced significant liver damage. No rats died throughout the trial in group I, but six died in group II. However, the number of animal deaths in the test group was lower than in group II. The CCl_4 administration caused significant hepatic necrosis, ballooning hepatocyte degeneration, and inflammatory cell infiltration, according to the results of the histological investigation with H&E staining in (Fig 8).

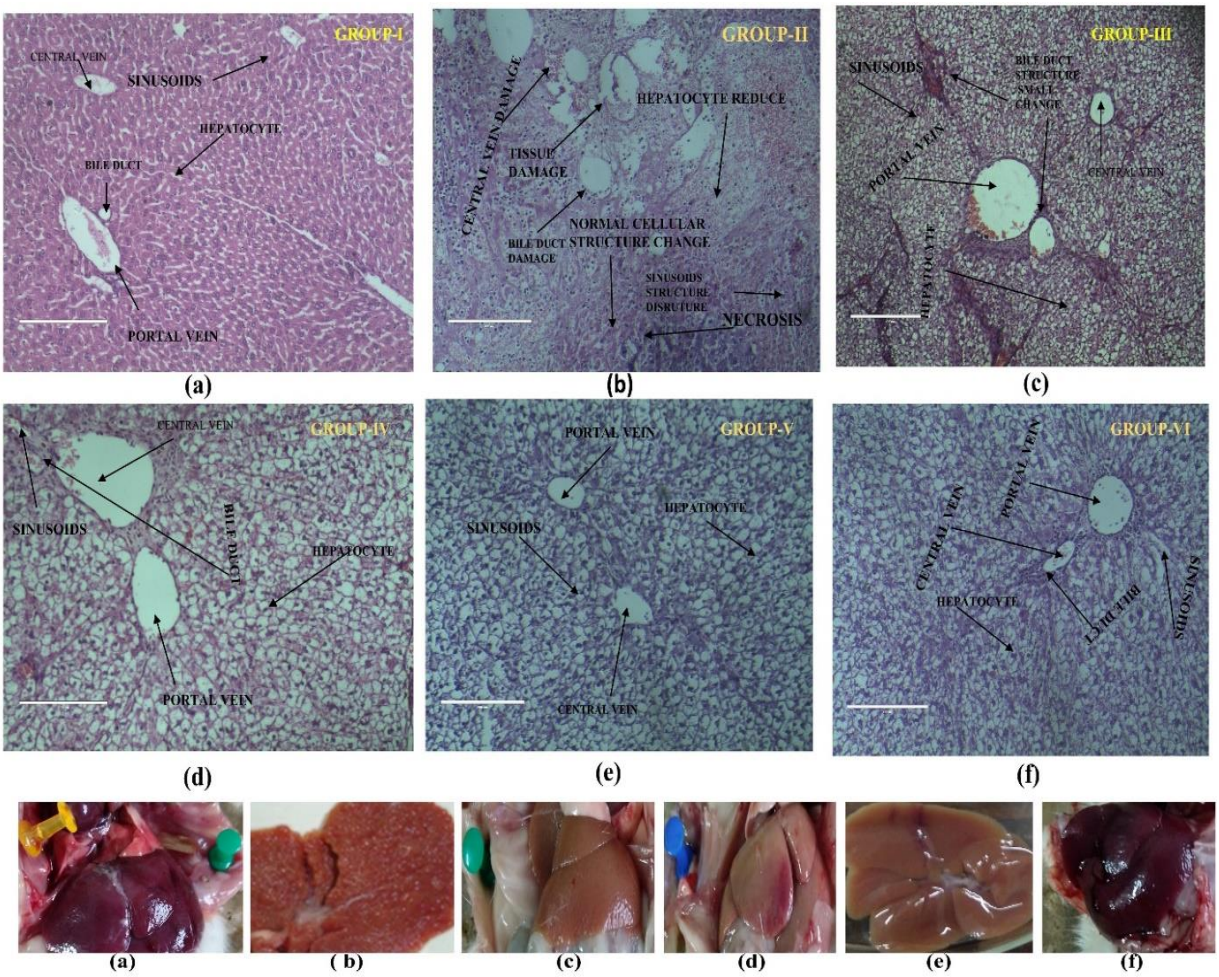

Figure 8. Histopathology of the liver from Group I (Fig 8a) control rats showing normal histological structure (bile duct, portal vein, and hepatocyte) was completely normal—histopathological image of a Wistar rat liver (H&E) 20x. Control Group I (Fig 8a) shows the Wister rat liver sections characterized by normal hepatic sinusoids, hepatic zonation, central vein, no fibrosis developed, and proper-shaped hepatocytes. Group II (Fig 8b) displaying marked distortion of hepatic architecture, fibrotic tissue, hepatocellular necrosis, ballooning degeneration, cytoplasmic vacuolization of the hepatocytes (black arrows), sinusoidal dilatation, congestion (white arrows), and inflammatory cell infiltration (black heads), destruction of hepatocytes, zonation change due to hypoxia, iron deposition, and bile duct hyperplasia; these changes were necrotic and degrading. Hepatocyte disruption was evident in Group II (Fig 8b) compared to the control group (I). some fat change from the central vein congestion little developed—group III (Fig 8c) No fibrosis-occurring sign developed or little structural change, and the central vein did not change shape in the group IV, V, and VI (Fig 8d, 8e, & 8f). also shows a greater reduction in hepatic injury than Group II.

5.8.12. Ethanol effect blood plasma deoxycholic acid (DCA) & chenodeoxycholic acid (CDCA):

In this study rat blood plasma was analyzed by HPLC. No significant increase in CDCA and DCA was observed in group I. But group II showed a significant increase in percentage of DCA and CDCA from group I. This experiment was fully performed at two independent bile acid concentrations. In (Fig 9E) shows the percentage increase of CDCA in rat plasma by HPLC analysis of groups I, II, III, IV, V&VI that were 14.56%, 64.86%, 59.92%, 53.37%, 50.65 and 46.90% respectively. (Fig 9F) shows that the HPLC chromatogram and the percentage increase of DCA in rat plasma by groups (I, II, III, IV, V & VI) as 8.3%, 58.74%, 55.92 %, 55.91 %, 45.37 %, 42.65 %, and 40.9 % respectively.

5.8.13. HPLC method validation for EtOH induced heepatotoxicity:

Liquid–liquid extraction (LLE) technique was adopted for the recovery of analytes from blood plasma in this study. Its concentration (0.05-0.8 ug/ml) and value was obtained with a correlation coefficient (r^2) of 0.997. The limit of detection and limit of quantification were estimated to be 30.8 and 50.8 ng/ml, respectively. Intra and inter-day precision were within 15.0%. The results of this study showed good extraction efficiency.

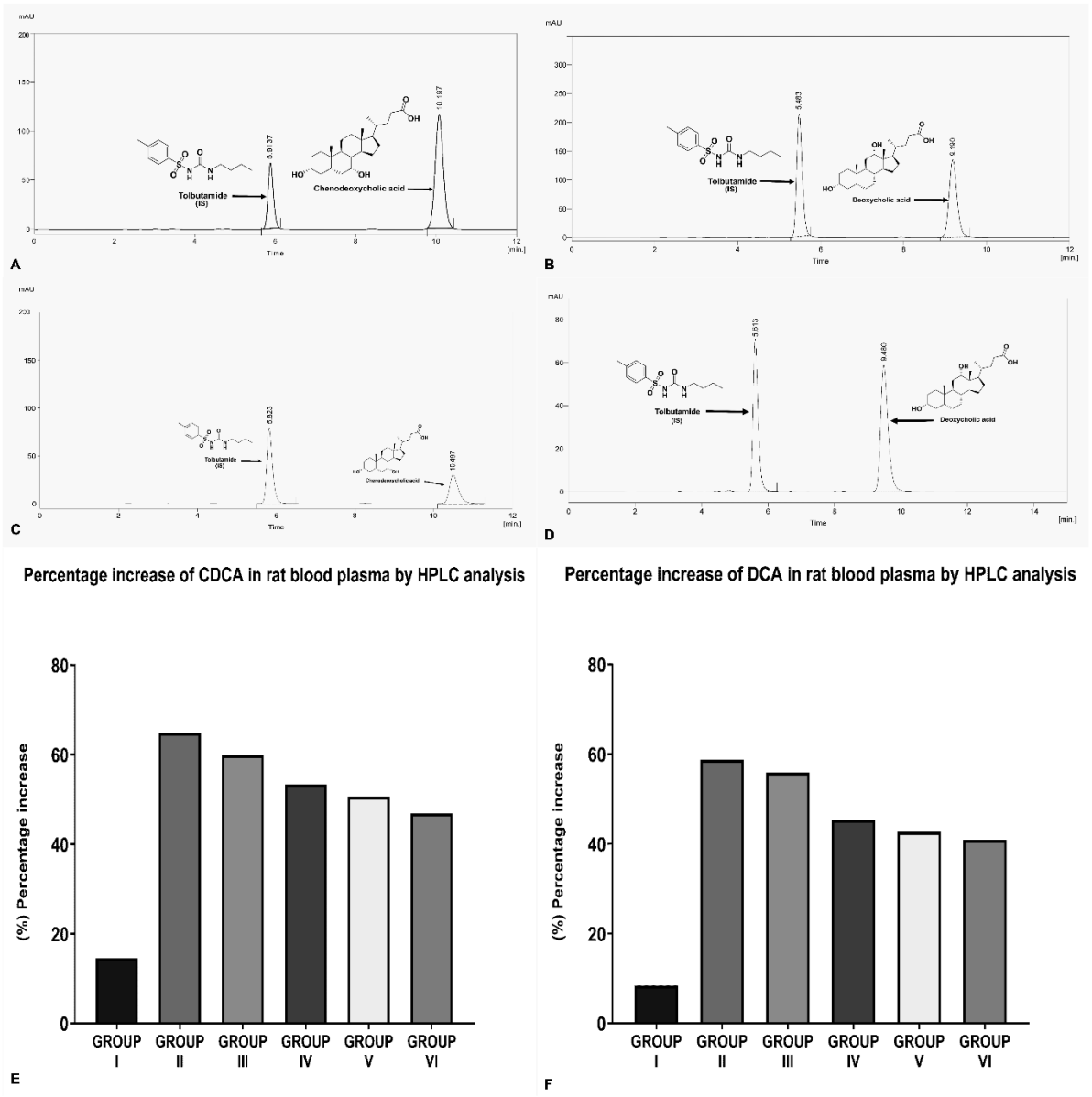

Figure 9. (A, C) showed a chromatogram of chenodeoxycholic acid (CDCA) and (**Fig** 9B, 9D) showed a chromatogram of deoxycholic acid (DCA). (**Fig 9A, 9B)** Chromatogram obtained from Et-OH induced hepatotoxic group. (**Fig 9C, 9D)** Chromatogram obtained from MP+GM treated group. Percentage increase in rat plasma by HPLC analysis groups (I, II, III, IV, V&VI) respectively 14.56%, 64.86%, 59.92%, 53.37%, 50.65 and 46.90%. (**Fig** 9E) and (**Fig** 9F) showed a chromatogram of chenodeoxycholic acid (CDCA) and showed a percentage increase in DCA of rat plasma by HPLC analysis groups (I, II, III, IV, V&VI) respectively 8.3%, 58.74%, 55.92%, 55.91%, 45.37%, 42.65%.

5.9.1. LC ESI MS/MS analysis blood sample of cholic acid in CCl4-induced hepatotoxicity

Rats were given MP in treatment groups, where it was observed that MP treatment from group II resulted in cholic acid (CA) returning to the previous level which was more like group I i.e. normal. Our study showed that LC-ESI MS/MS analysis of CA was statistically obtained in rat plasma samples, which corresponded to disease control group II. The percentage of increase in the group II caused by CCl_4 intoxication was 77.94%, while the normalization in the experimental group was 59.92%, 49.37%, 42.77% and 23.8%, respectively. All experiments were performed in triplicate.

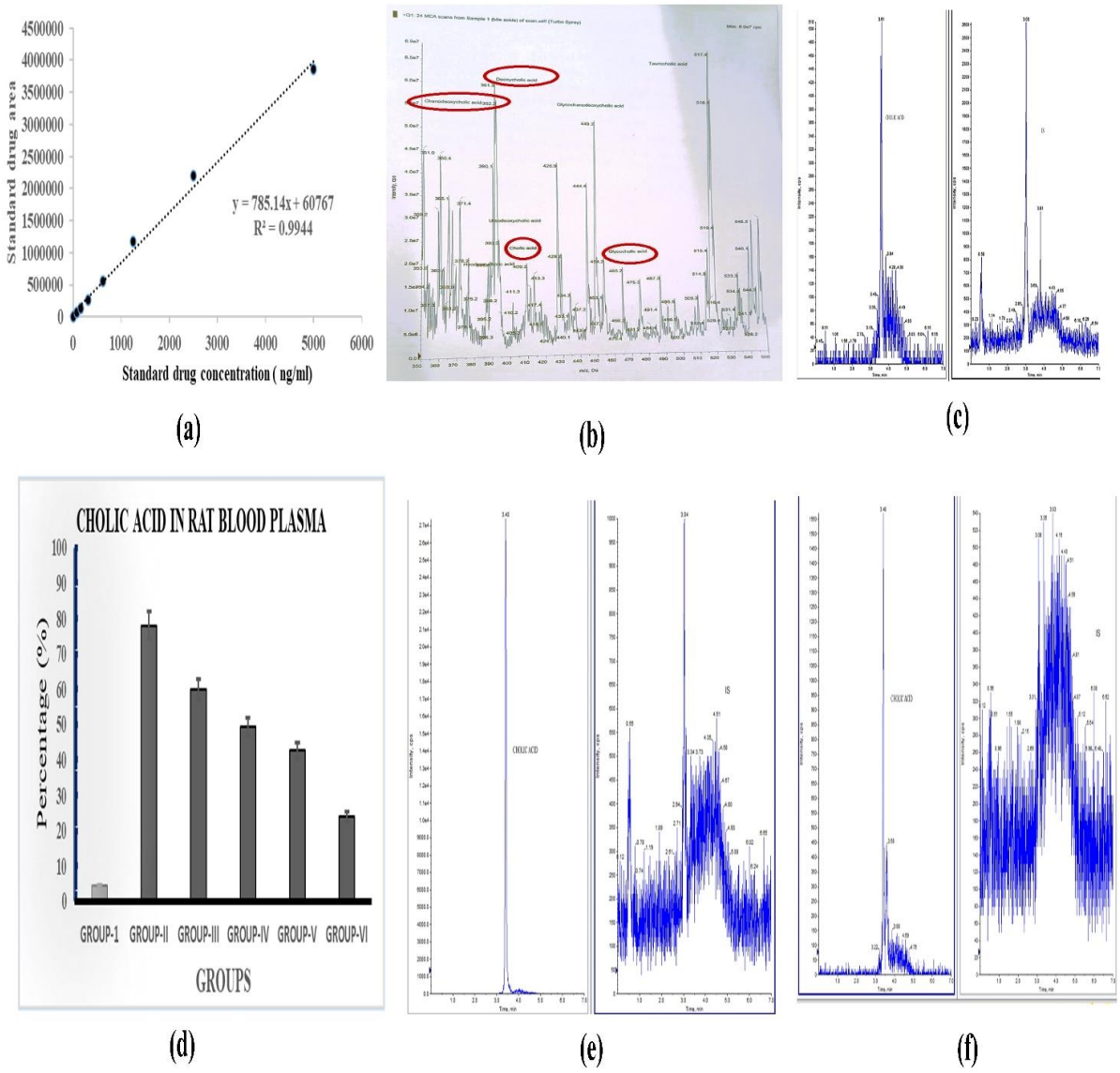

Figure 10. The quantitative measurement of cholic acid in rat plasma from groups I, II, III, IV, V, and VI was assessed using a standard curve (Fig10a). Within group II, it was noted that only the use of CCl_4 caused to an elevation in the percentage of cholic acid, as seen in (Fig 10d). (+Q1) and MCA scans from blood plasma Sample (bile acids) of scan (Turbo Spray) (Fig 10b).red marking were identified mass of cholic acid, chenodeoxycholic acid, deoxycholic acid in (Fig 10b). the quantification of blood plasma cholic acid mrm (c), (e), and (f) in (Fig 10).

5.9.2. Fast protein liquid chromatography (FPLC) protein purification chromatography:

Here, FPLC is run with standard proteins to see that larger proteins elute earlier due to shorter retention times. But since the molecular weight of our proteins is low, they are coming out at the very last. The elute of each purified protein is automatically collected in a 2 ml ependrop. Peak numbers 24.22 (Fig 11) were then collected for SDS-PAGE analysis .

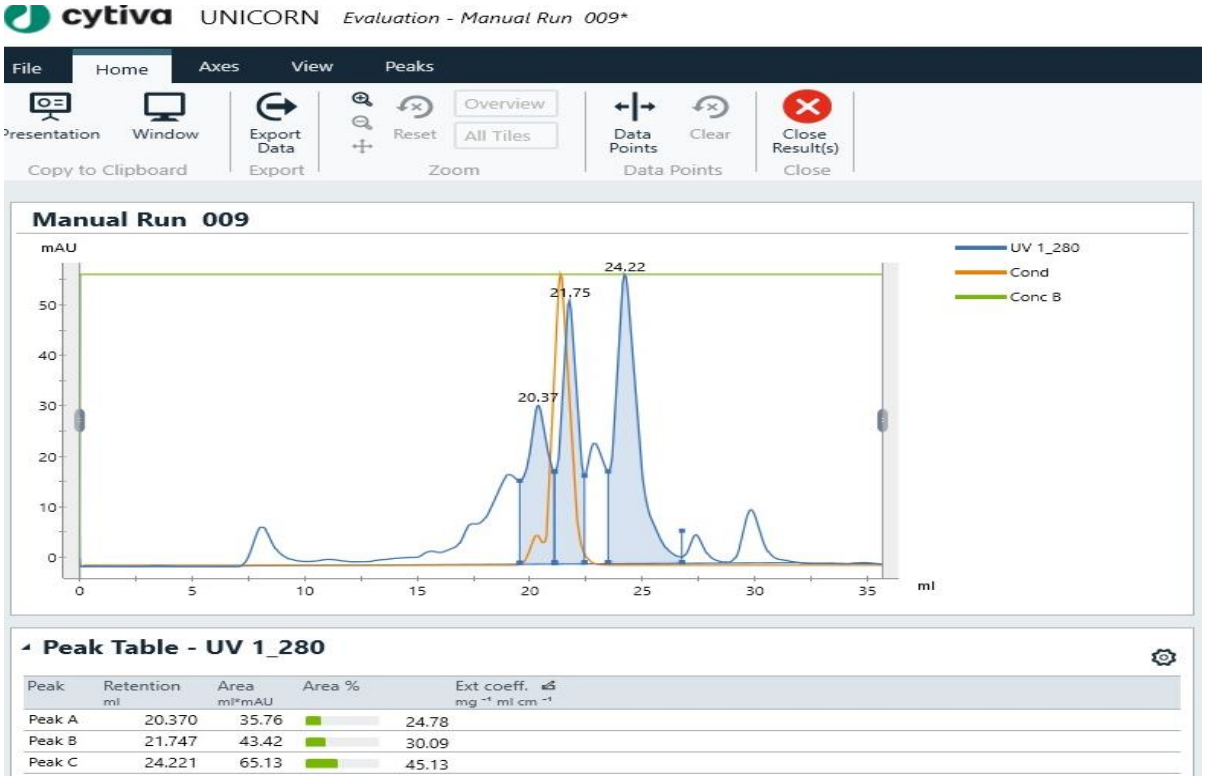

Figure: 11. FPLC(cytiva unicorn) separation of MP proteins by Superdex 200, and lastly, HiTrap SP HP prepacked column chromatography. Proteins are fractionated by 5HiTrap SP HP prepacked column chromatography, and elution is monitored by absorbance at 280 nm.

5.9.3. Purification of Protein:

Indian earthworm *Metaphire Posthuma* crude extract was prepared by autolysis in a 50 mM phosphate buffer at pH 7.5. The crude extract was subjected to salt precipitation, and 70% ammonium sulphate was used to recover the total protein. The precipitate's protein content (1.34 and 0.92 mg/ml) was reported. Using a combination of phosphate buffer and KCl, 25 separate fractions were collected from the protein on 5-ml HiTrap SP HP prepacked columns. The concentration of purified protein reached 0.0780 mg/mL. Pooling fractions no.24 and loading onto a 12% polyacrylamide gel with a standard protein ladder, the average molecular weight was 6 kDa, as determined by SDS-PAGE with a standard low-range protein ladder and confirmed by MALDI-TOF-MS in (Fig 12).

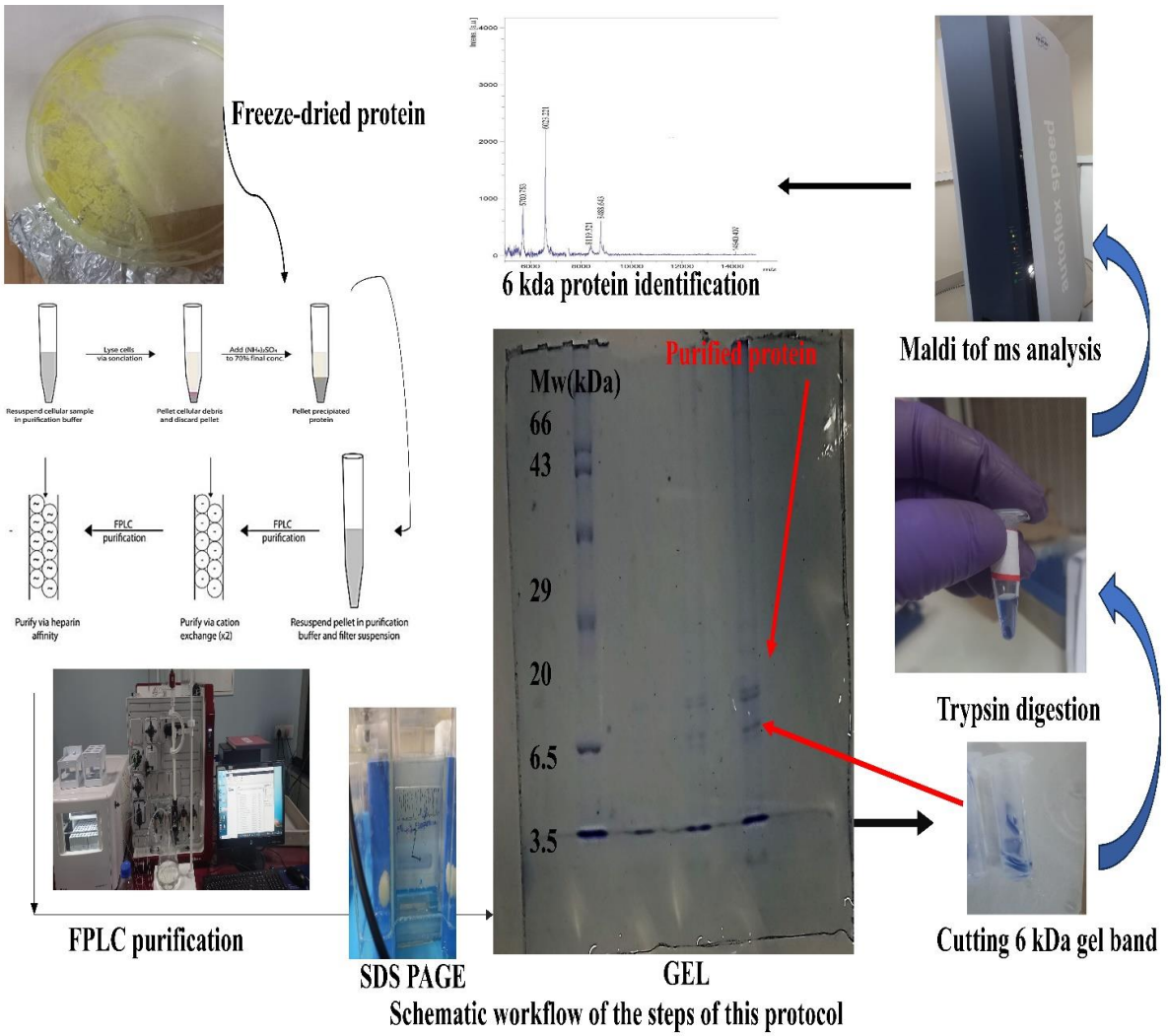

Figure: 12. Here, we freeze-dried powder protein salting-out process extraction protein, which was used for a fast purification process in FPLC. Then, SDS-PAGE was performed for purified protein identification. Below 10 kDa, mainly 6 kDa protein cut off for in-gel (1D gel) digestion or trypsin digestion, and MALDI TOF (Bruker) is used for molecular weight and peptide identification by mascot analysis.

5.9.4. Protein characterization by mass spectrometry after trypsin digestion

In-gel-digesting, or trypsin digestion, of proteins extracted by gel electrophoresis inside a gel matrix is a fundamental technique in proteomics research that utilises mass spectrometry (MS) analysis. Maldi-ToF MS spectra were constructed utilising the m/z ratios of trypsin-digested, purified protease fragments. These results supported previous investigations of *Metaphyre posthuma* species that indicated metallothionein-2 as the smallest protein picked up by trypsin digestion from SDS PAGE of 6 kDa (1D) gel of the lowest level of purified protein from earthworm extracts.

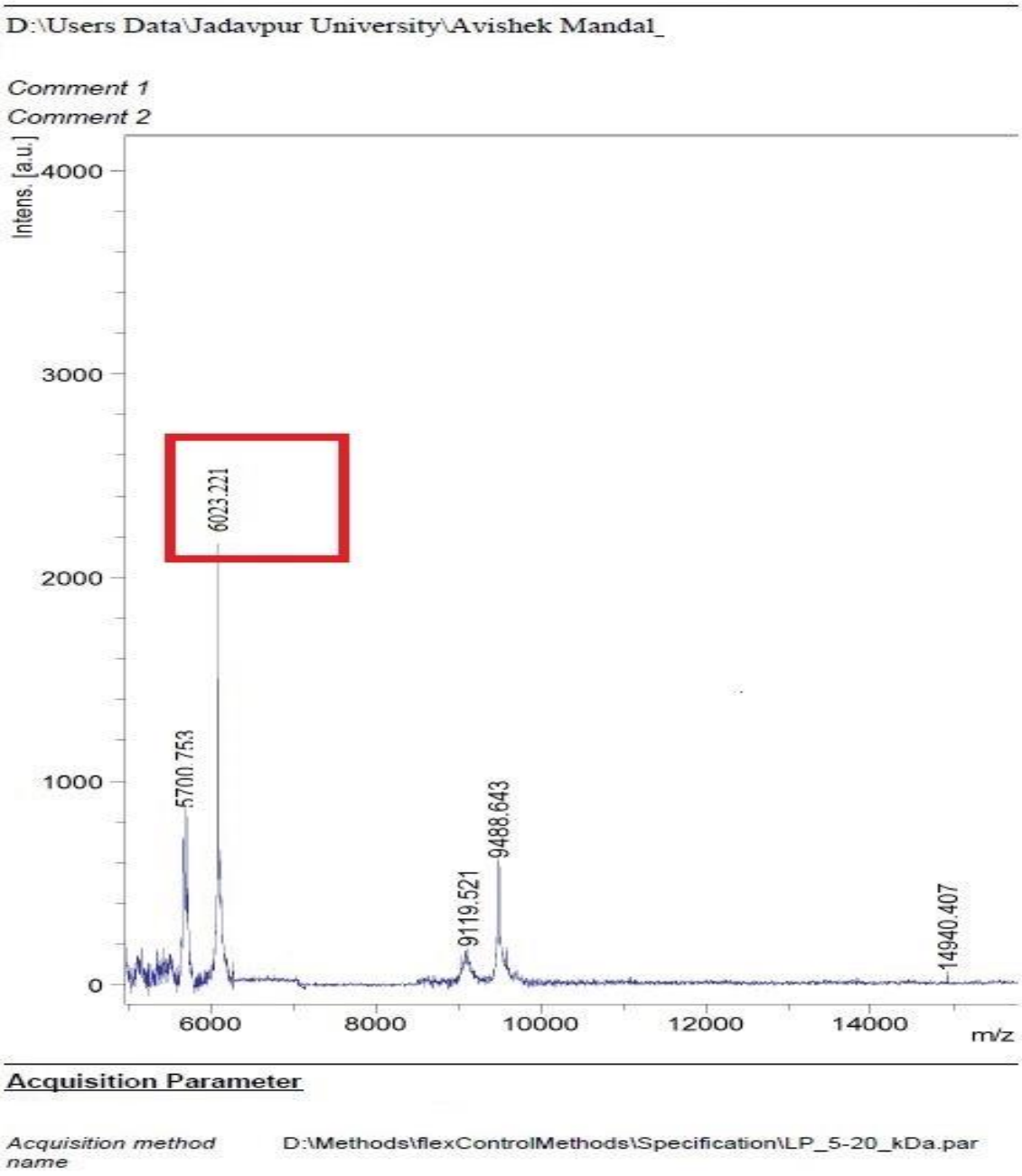

Figure 13. A mass study of purified protein for molecular weight determination. The protein was reported at 6023.221Da (red marking) through MALDI-TOF mass spectrometry study.

Table 6. Purification and quantification summary of Earthworm protein *Metaphire Posthuma*

Purification	Concentration of protein (mg/ml)
Crude extract	5.4
Salt precipitation	3.94
Dialysis	0.86
Sephadex G75	0.64
MWCO 50	0.37
FPLC purification	0.014

Table 7. Amino acid sequence and Metallothionein II peptide

Sl no.	m/z	Amino acid sequence	Molecular function
1. Metallothionein (II) 2. Metallothionein (II) 3. Metallothionein (II) 4. Unknown	1059.2303 634.4684 3293.2411 671.5326 3293.2411 1783.3425	ECLPNCDKNCCGTGCGSAKCGNASCKCGAD MSDNTKCCGK PACPREDSKC CKCGADCKCTGGPTC GGPTCATDCA VCPNCQCAKG ECLPNCDKNC CGTGCGS VFAFSKJXFDSFDSSKKPGCGGECPL	Metal binding, heavy metal detoxification, cellular antioxidative defense, hepatocyte regeneration

An MS/MS study of purified and trypsin digested earthworm metallothionine II proteins Selected masses were subjected to de novo sequencing and database searches from matrix database by mascot analysis software which indicates similarity among characterised proteins only fifty percent similarity with metallothionine II according to NCBI database.

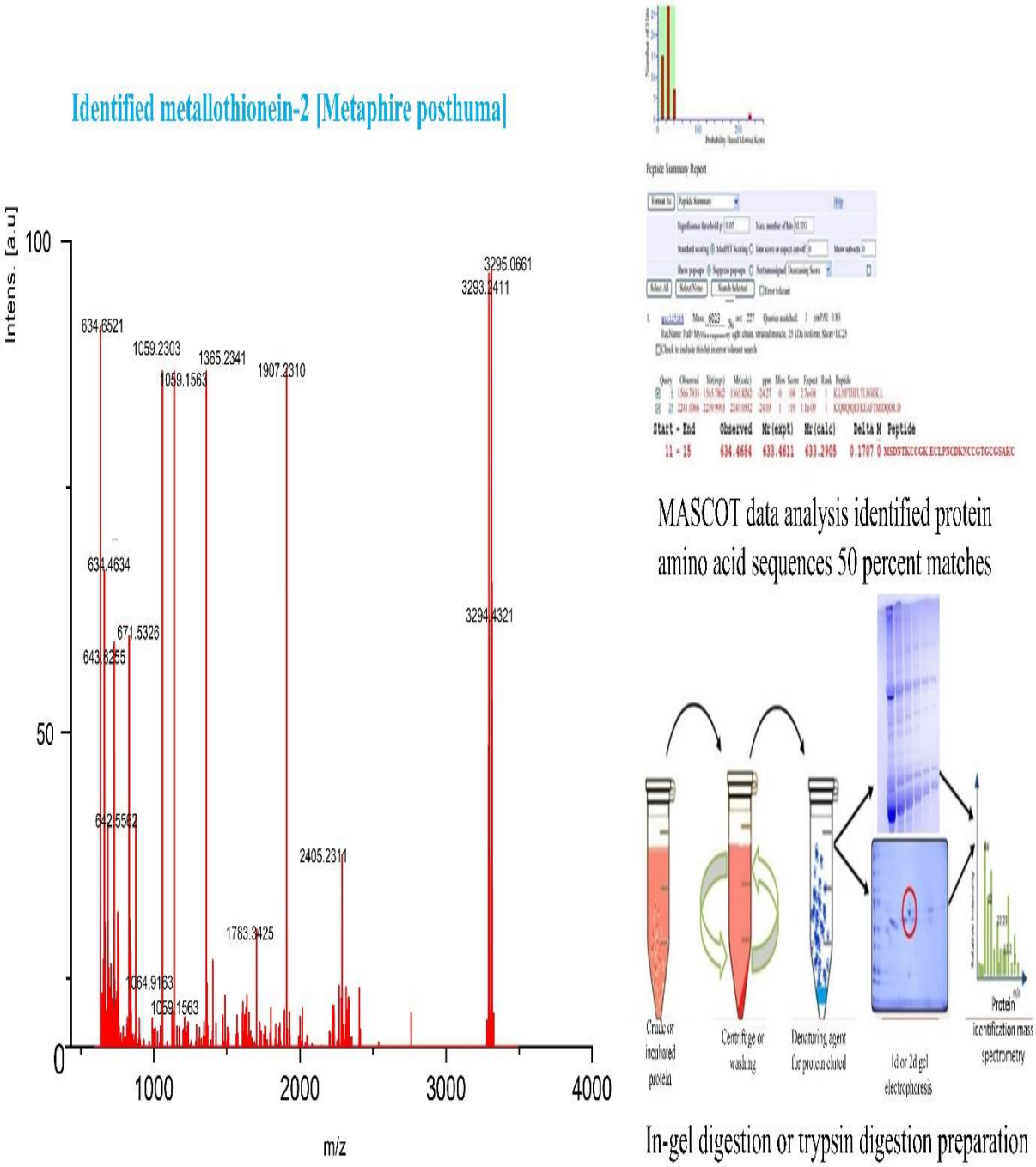

Figure 14. MALDI–TOF mass spectrum analysis of trypsin digested purified found Earthworm metallothionine-2 peptide by mascot software analysis . A) MS spectra. MS/MS analyzed the marked ion 1059.2303, 634.4684, 662.4402, 3293.2411 and 1059.6742.

Discussion

Drug-induced liver damage may cause acute or chronic hepatic diseases, or both [34-37]. Alcohol consumption can also contribute in the development of cholestasis by causing liver injury, oxidative stress and inflammation, which can lead to bile duct obstruction or damage [38]. Cholestasis, which is a reduction or blockage in the flow of bile from the liver to the intestine can potentially affect on alcoholic liver damage. Cholestasis can progress to alcoholic hepatitis, which is a more severe form of liver disease that is associated with significant morbidity and mortality [37-38]. Alcoholic hepatitis, lipid accumulation and faster development of liver disease as well as a greater incidence of cirrhosis are all conditions that have been linked to chronic and acute alcohol ingestion [39]. The present study examined body weight, liver function activity AST/ALT, cholesterol, triglycerides, anti-oxidant activity, inflammatory marker and bile acid expression mainly deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA), in EtOH-induced rats treated with MP and GP. Literature studies indicated that DCA and CDCA have lipophilic properties, which can cause toxicity in the primary stages and an indicator of hepatitis during the progression of liver damage. The imbalance of bile acids in liver damage can lead to an elevation in their percentage, which is a contributing factor to the initial damage seen in cholestasis. The quantification of CDCA and DCA is viewed as one of the more sensitive and convenient methods for identifying liver damage. The levels of these two bile acids in the serum are vital markers for the clinical diagnosis of liver disease and other related conditions [40]. Considering the rising therapeutic risks associated with natural products [41–43], this study was conducted to assess the safety of MP through a single-dose oral acute toxicity test, using 2000 mg/kg as the maximum dose. Over a 14-day observation period, no mortality or significant clinical signs of toxicity were observed in either the control or MP-treated groups. Furthermore, no significant changes were noted in relative organ weights (Table 1) and in the body weights of the animals (Table 2), suggesting that the maximum tolerated dose of MP in rats could be higher than 2000 mg/kg. A sub-acute toxicity evaluation of MP was also carried out through a 28-day repeated dose (500, 1000 or 1500 mg/kg/day) toxicity study in rats to assess its endocrine-related effects, histopathological parameters, and the no observed adverse effect level (NOAEL) [44]. Body weights, complete serum biochemical profiles, and histopathological examinations were conducted to evaluate potential toxic effects of MP on viscera. No noticeable abnormalities were observed during histopathological studies, and there were no

significant changes in the relative weights of vital organs such as the liver, kidney, spleen, and heart, between the control and MP-treated groups. Serum biochemical parameters, including creatinine and urea, which are important renal toxicity biomarkers, were also examined [45][46]. Apart from reduced AST and serum sodium levels, no significant differences were found in ALT, glucose, triglycerides, urea, and creatinine levels in the MP-treated groups (Table 4). This suggests that oral administration of MP does not produce any toxic effects in internal organs. This was further confirmed by histopathological examination results, which showed no obvious changes in the liver, spleen, and kidney compared to the control dose, even at the maximum dose of 1500 mg/kg/day (Fig 1). According to the results in Et-OH challenged group (Group- II), the body weight and liver weight was found to be 154 g and 6.2 g respectively which suggests a notable decrease in body weight and liver mass in animals, compared to the control group (body weight 197g and liver weight 8.4g). This is attributed to poor nutrition from daily alcohol intake. Alcoholic liver disease presents issues such as glutathione depletion, decreased levels of albumin, and liver damage. Ethanol (EtOH) is linked to increase mortality and abnormalities in hepatocytes. Higher alcohol doses exacerbate liver damage and can interfere cell growth. Generation of free radicals from EtOH hinder the hepatic health, inhibiting hepatocyte proliferation and increasing mortality, especially in the EtOH challenged group. Interestingly, the MP-treated group IV showed promising results when compared with the EtOH-treated groups in terms of body weight gain (154 to 171g). Specifically, the protein derived from earthworms, which is rich in amino acids, proteins, and anti-oxidants, along with several bioactive component, has substantial nutritional value [47]. This effect led to an increase in body weight among animals in both the GM and MP-treated groups (147 to 177 g). In addition to identifying microvascular steatosis and mild inflammation, the treatment of group II with EtOH resulted in apoptosis, necrosis, and congestive hepatopathy in cellular structures. Another problem associated with alcoholism is the AST/ALT ratio. A high ratio indicates recent exposure or severe alcoholic liver damage [48]. In this study, group II showed a similar increase in AST /ALT ratio. Where the percentage of inhibition was 32.98%, 51.05%, 76.62%, 80.70% respectively in the treatment group III, IV, V, and VI respectively. Cholesterol, a key component of cell membranes, interacts with lipoproteins and is a steroid hormone precursor [49]. EtOH administration increases fatty acid synthesis and reduces hepatic lipoprotein release, lowering blood cholesterol [50]. Reduced cholesterol absorption could increase liver bile acid conversion, inhibiting cholesterol synthesis and suggesting potential hepatobiliary issues in

EtOH intoxication. Results from this study suggest that EtOH reduced cholesterol levels in group (II) animals, aligning with prior *in vivo* studies [51]. Although cholesterol was reduced in group II, triglyceride levels were significantly increased (at 101 mg/dl). The percentage of inhibition due to treatment was 15.75%, 28.75%, 30.07% and 39.84% in groups (III, IV, V and VI) respectively (Fig 6). EtOH challenged group (II) also raised triglycerides due to its hepatotoxic effects. Despite an initial cholesterol decrease, chronic alcohol use typically elevates cholesterol levels [52]. However, the treatment group (III, IV, V and VI) maintained moderate cholesterol levels. Group III, IV, V, and VI normalized total blood cholesterol at 4.41%, 11.60%, 40.33% and 43.64%; and reduced triglyceride levels by 15.78%, 28.57%, 30.07%, and 39.84% with respect to group I. The hepatoprotective properties could be linked to its complex protein content and anti-oxidant properties [53]. Treatment of groups III, IV, V and VI resulted in improved serum cholesterol concentration in our experiment, which was very similar to that of group I. Thus, MP-treated rats controlled EtOH-induced liver triglyceride and cholesterol in Fig. 02. While, the enzyme CYP2E1 has an unique ability to metabolise and activate several substrates that have toxicological significance, such as ethanol, carbon tetrachloride, and acetaminophen. CYP2E1 is a key factor in the production of oxidant stress in the liver due to ethanol [54]. The metabolism and oxidative stress enzyme CYP2E1 quantification is unique in the liver toxicity test. The CYP2E1 levels in Group II (473.66 ng/ml) increased significantly from Group I (197.83 ng/ml). Results for treatment groups V and VI (362.5 and 308.5 ng/ml) were found significantly lower ($p < 0.05$) than Group II in fig.03. Cell viability is crucial for tissue regeneration, enhancing division, repair, stem cell activity, extracellular matrix production, and modulating inflammation. It also allows cells to utilize nutrients and oxygen effectively. Furthermore, findings from the trypan blue exclusion assay on primary hepatocyte suggested that the percentage of viable cells were higher in groups V and VI (73% and 81%) than in group II (47%) which hypothetically points in the direction of primary hepatocytes carrying on their regeneration process in (Table 5). Alcohol can also alter hepatic BAs balance by changing the intestinal microbiota and boosting BA production [55][56]. It also aggravates conditions like cholestasis and that are linked to disruptions in BA production and absorption [57]. In this study, the results showed EtOH consumption caused liver damage by increasing the AST/ALT ratio and DCA levels in serum. Recent studies suggest that CDCA, and DCA can be toxic when overproduced in the liver [58]. The experimental data showed that the levels of hepatotoxic DCA and CDCA were significantly higher in group (II) rats (58.74% and 64.86%), serving as potential indicators of

alcohol-induced liver damage. Alcohol consumption generally increases free BA levels, particularly toxic ones, contributing to liver damage [59]. Since studies have showed that the retention time of bile acids (CDCA & DCA) is between 8-15 min [59]. This study showed that the retention time of bile acid (CDCA and DCA) was 10.197 and 9.190 min. As shown in (**Fig 9A**) the retention time (RT) of high-level CDCA in the blood plasma of group II showed an increase in the area under the curve at 10.197 min compared to the internal standard. But it was seen to decrease in the treatment group in (**Fig 9C)**. Similarly, as shown in (**Fig 9B),** the RT of DCA is 9.190 min. and the area under curve was found to be increased in group II. While in the treatment group, it was found to be decreased, as in (**Fig 9D)**. Thus, treatment with GM, MP, and GM+MP resulted in lower serum levels of DCA and CDCA than in the EtOH-treated group respectively (55.92%, 55.91%, 45.37%, 42.65%, 40.9% and 59.92%, 53.37%, 50.65, 46.90%).

In this CCl_4 induced hepatotoxicity model study, crude and purified earthworm proteins were extracted. The crude protein was obtained using the salting-out process. On the other hand, SDS PAGE analysis was performed after purification of earthworm proteins from protein dialysis, Sephadex-g75, 50 kDa MWCO, and FPLC (Fig 11,12). The sequence of some peptides from the 1D (SDS PAGE) protein band is then determined using the amino acids' sequence in-gel digestion mass spectrometry technique. The extracted protein bands were all less than 10 kDa. As a result, we detected the 6.2 kDa band proteins with a low molecular weight expected to have greater therapeutic efficacy in (Fig 13). However, the NCBI database reveals that metallothionein 2 is particularly abundant in the low-molecular-weight protein of *Metaphire Posthuma* species (MP). It is a liver antioxidant that increases regeneration and prevents hepatocyte metal toxicity [61]. Scar tissue is formed as a result of excessive extracellular matrix protein accumulation in the liver. It may be caused by toxic exposure to chemicals and heavy metals. Several investigations on the impact of metallothione in gene therapy on liver fibrosis have shown that metallothionein in reduces oxidative stress, metal ion binding, antioxidant activity, and cell regeneration potential [61]. MALDI-TOF-MS in gel digestion or trypsin digestion revealed the presence of metallothionein-2 in the 6.023 kDa band in our investigation in (Fig 14). Despite the fact that this was merely a qualitative analysis, in (Fig 14). it might be considered that metallothionein-2 performed a key role in protecting the liver against CCl_4. Earthworms exhibit a variety of pharmacological activities in various traditional Chinese medicines, but

evidence evaluating pathways for liver damage or disease amelioration still needs to be unveiled [62]. In this study, we looked at MP potential efficacy and effectiveness in managing CCL_4-induced liver fibrosis. The development of hepatic injury associated with oxidative stress, lipid peroxidation, and cell degeneration leads to hepatic fibrosis. Ultrastructural changes, steatosis, and inflammation are early signs of liver pathology, followed by hepatitis, dysplasia, and necro-inflammation in later stages. According to the pathological investigation, the disease control group showed cell necrosis, fibrous tissue hyperplasia, internal enlargement, and pseudo-lobule replacement of standard lobular architecture in CCl_4-induced hepatic injury. Compared with the CCl_4 group II, groups treated with MP with CCl_4 toxicity simultaneously showed limited hepatic cell necrosis, fewer interstitial collagen fibers, and limited lobular structure injury in (Fig 8). Here, we used below 50 kDa MCOW extract purified protein from earthworm extract obtained from the salting-out process, then gel filtration and dialysate membrane filtered for extra salt elimination and further lyophilized to powder. The purified protein concentration found in the Lowry protein assay and SDS page was subjected to purified protein identification, as shown in (Fig. 3B,3C). Our data revealed that earthworm extract protein (50, 100, and 200 mg/kg) and CCl_4 simultaneously for 12 weeks limited the development of liver fibrosis. Compared to the disease control group, treatment groups III, IV, V, and VII showed reduced serum STAT3, IL-4 and increased total protein, treatment groups. In contrast to the disease control group II, the histology of the liver was also significantly improved in (Fig 8). Following this study, we hypothesis that MP may act through multiple pathways to protect liver fibrosis. The application of CCl_4 triggers mitogenic signaling, which results in the release of antigens. This causes CCl_4 to act as a chemoattractant for natural killer cells, monocytes, and other immune cells at the site of inflamed or damaged tissue [63]. Consequently, this leads to the release of various pro-inflammatory cytokines. The current study also found that MP greatly suppressed the STAT3 signaling [64] pathway that was activated by CCL_4 (group II) in the liver of rats. IL-4 levels in cases of chronic hepatitis have been the subject of conflicting reports. While some researchers observed reduced IL4 levels in HCV patients, others found these levels to be noticeably higher [65][66]. However, in our study, the disease control group's IL4 increased but was not significant in the treatment groups (III and IV). The preventive effect of EEP against CCl_4-induced hepatic fibrosis in rats involved STAT3 inhibition. Several cytokines, growth factors, and hormones can activate the JAK1/STAT3 signaling pathway, which is crucial for developing hepatic damage or fibrosis [66][67].

Activation of STAT3 has been seen in several liver conditions, especially hepatocellular cancer, steatosis, fibrosis, and liver damage [68][69][70]. Our previous research, consistent with the present one, demonstrated that MP effectively suppressed paracetamol-induced inflammation by inhibiting the IL-6 pathway. In this study, inhibition of the STAT3 signaling pathway was accompanied by a decrease in IL-4 expression, which indicates the suppression of inflammation [71]. Hypothetically in this case it can be said that MP helps to reduce inflammation and increase the protection of the liver. According to several studies, IL-6 stimulates STAT3 in HSCs and ensures their survival, proliferation, and activation, which results in hepatic fibrosis [72]. The STAT3 pathway was reportedly activated in liver fibrosis, and the levels of STAT3 in this study were substantially higher in group II compared to the treatment MP (groups III, IV, V). STAT3 levels in Group II (110.01 pg/ml) increased significantly from Group I (24.63 pg/ml). Results for treatment groups V and VI (56.47 and 42.94 pg/ml) were found significantly lower ($p < 0.05$). On the other hand, in the case of IL-4, it is seen that group II significantly increased from 2 pg/ml to 48.14 pg/ml from control group I. On the other hand, group III (43.5 pg/ml) and IV from group II. (35.5 pg/ml) did not show significant level change. But group V (25.4 pg/ml) and VI (20 pg/ml) significantly reduced showed in (Fig 4). Continuous weight loss was seen in CCl_4-induced chronic liver damage rats, whereas MP prevented weight loss in the therapy groups (Fig 4k). In addition, we discovered that treatment groups improve survivorship. The development of hepatic fibrosis is strongly associated with stellate cell proliferation and differentiation. Due to CCl_4 toxicity, α-SMA expression is significantly elevated in chronic liver damage as a result of stellate cell over-activation [73][74]. Recent investigations have suggested that α-SMA regulates stellate cell transcription in liver damage. Stellate cell activation plays an important role in the pathogenesis of fibrosis [75][76]. Alpha-SMA expression level was significantly lower in group IV, V, and VI when compared with group II of our animals *($P < 0.05$), **($P < 0.01$) but here the change in group III was found non-significant fig.05. Several studies have recently demonstrated that the PI3K/AKT/mTOR signaling cascade leads to HSC activation and proliferation and changes in ECM composition, tends to progression of liver fibrosis [77][78]. Surprisingly, in our study, the mRNA expression of AKT1 was found less in the treatment groups compared to the control group II. Although no significant change was found in group II while compared with group III, but significantly changes were observed in group IV,V and VI when compared with group II *$p<0.05$, **$p<0.01$. Different signaling cascades, involving mitochondrial apoptotic pathways are responsible for cell death. The Bcl-

2 family of proteins regulates the integrity of the mitochondrial outer membrane in various stages [79]. This family includes both pro-apoptotic (Bad and Bax) and anti-apoptotic (Bcl-2 and Bcl-xl) proteins [80]. Additionally, CCl_4 treatment upregulated Bax mRNA expression in control group II, but we observed MP treatment in groups III, IV, V and VI significantly reducing fig.05.compared to group II ***($p<0.001$). The fact that the Bax protein directly links with the degree of liver fibrosis suggests that Bax is only essential during the early stages of liver fibrosis [81]. NRF2 is a major signaling molecule with antioxidant activity and is crucial for controlling a network of genes involved in upholding redox states and host defence mechanisms, both of them are necessary for hepatocellular health [82][83]. According to one research, NRF2 activators have antioxidant action, which may lead to a reduction in liver fibrosis [84]. In our current investigation, MP elevated the mRNA expression level of nuclear NRF2 while compared to the disease control group II. Biliary fibrosis is the main pathogenic mechanism that causes cholangiopathies, including primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) [85]. Cholangiopathies are linked to cholestasis, a condition characterised by the accumulation of biliary components, such as bile acids, in the liver and bloodstream [86][87]. Literature studies have shown that cholic acid has lipophilic characteristics, which may lead to toxicity in the early stages of hepatitis as liver damage progresses [88]. Several investigations have shown that cholic acid is abnormally increased for various bile acids tested after CCI_4 administration. In particular, 7-keto DCA, GUDCA, and glycine-conjugated CA and CDCA demonstrate an association with progressive fibrosis [89][90][91]. The experimental data showed that the levels of hepatotoxic CA were significantly higher in group (II) rats (77.94%) (Fig 10), serving as potential indicators of CCl_4-induced liver damage. CCl_4 consumption generally increases free BAs levels causing high toxicity that contributes to liver damage [92]. However, treatment with MP, resulted in normal serum levels of CA than in the ccl4-induced group respectively (59.92%, 49.37%, 42.77% and 23.8%) in (Fig 10). Thus it could be inferred that MP is effective against liver toxicities induced by different agents. It is safe to assume that consumption of MP may be useful in different liver disorders, if utilised properly.

Summary

The results of our study on the effects of MP on paracetamol-induced hepatocellular damage align with earlier statements on the reduction of oxidative stress and inflammation. The taxonomic classification of the earthworms previously mentioned are same ones examined in our study (*Metaphire posthuma* previously known as *Pheretima posthuma*). The scientific relevance of MP (*Metaphire posthuma*) activities lies in their ability to induce cytokine expressions, such as TNF-α and IL-6. These activities entail using smaller protein fractions having a molecular weight of less than 50 kDa from the dialysate. These protein fractions are directly applicable in real-time scenarios. This study will enable us to further investigate and identify a more effective mix of earthworm protein fractions that may be used as a hepatoprotective product.

In some rural traditions of India, particularly in certain regions of West Bengal and Tamil Nadu, individuals suffering from liver disease are administered a remedy made by crushing earthworm extract and combining it with goat's milk. This research performed an in vitro test to determine the hepatoprotective activity of primary hepatocyte culture derived from the liver of rats. Among the available options are goat's milk (GP) at different quantities, as well as earthworm protein and goat's milk combined with earthworm protein. The current research revealed the potential hepatoprotective effects of both MP and MP+GP against liver damage generated by CCl_4. Additionally, it proved that the addition of GP boosted the hepatoprotective activity by lowering inflammation and oxidative stress. MP+GP often enhances liver regeneration and cell proliferation. In order to mitigate elevated levels of toxicity and oxidative stress, a combination of MP and GP is used. Hence, this bioactive MP, in conjunction with GP, has promise for being examined as a possible bio-therapeutic agent

for addressing human ailments, particularly acute or chronic liver injury. Bioactive proteins have several potential uses, particularly in the fields of pharmaceutical treatment and food.

Metaphire posthuma protein powder (MP) has been shown to possess notable hepatoprotective capabilities against ethanol-induced liver injury, after thorough examination and analysis. These advantages may be seen both when the powder is consumed alone and when it is combined with goat milk. Furthermore, MP not only demonstrates its safety even at greater dosages, but also has the ability to control lipid metabolism, restore antioxidant enzymes, and decrease inflammatory indicators. All of these advantages are attained without compromising its safety. One example of its many medicinal applications is its ability to reduce the harmful effects of bile acids on the liver. These results establish MP as a naturally occurring components with significant promise for future investigation in the area of liver function. This implies that it might be beneficial in therapeutic contexts.

To summarise, we successfully improvised a rat hepatotoxic model that was induced by CCl_4. The optimum therapy scenario for MP is to provide doses of 50, 100, and 200 mg/kg over a 12-week period. The MP exerted significant therapeutic benefits in the treatment of experimentally induced liver fibrosis.

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Avishek Mondal.

Sanmoy Karmakar 10/01/24

DR. SANMOY KARMAKAR
PROFESSOR (Pharmacology & Toxicology)
Dept. of Pharmaceutical Technology
JADAVPUR UNIVERSITY, Kolkata-32

Publications

Institutional Animal Ethics Committee (IAEC)

Department of Pharmaceutical Technology

Jadavpur University

Kolkata-700032

Ref No: AEC/PHARM/1702/3/2017

This is to certify that Project title **"Natural Remedies for Hepatic Diseases "**has been approved by the IAEC

Prof. (Dr.) Biswajit Mukherjee	Dr. Kuladip Jana
Name of the Chairman/ Member Secretary IAEC:	Name of the CPSCEA nominee
Chairman/Member Secretary of IAEC	CPSCEA nominee
Signature with date 13/12/17	Signature with date 13.12.17

Chairman
Institutional Animal Ethics Committee
Jadavpur University
Kolkata-700032

To,
Professor & Head Sanmoy Karmakar, **Date-24.02.2023**
Department of Pharmaceutical Technology
Jadavpur University, Kol- 700032, India
Email: sanmoykarmakar@gmail.com

Sub: Identification Report of Annelida (Earthworm) reg.

Ref: Id & Advisory Lot No. 3665

Respected Sir,

With reference to the above mentioned request, received - 2 vials of Annelid on 16 .02.2023. The specimens were examined and identified by me. The Identification report is as follows:

Phylum- Annelida

Class- Clitellata

Order- Opisthopora

Family-Megascolecidae

1. *Metaphire posthuma* (Vaillant, 1868). 1ex. Reg. No. An 8015/1.

Thanking you,
sincerely

Yours

(Dr. C. K. Mandal Scientist B)
Zoological Survey of India
27, J. L.Nehru Road, Indian Museum Campus
Kolkata-700016

THE PROTECTIVE ACTION OF EARTHWORM PROTEIN POWDER/ MWCO 50 KDA FRACTION ON EXPERIMENTAL HEPATIC DAMAGE: AN INVIVO/INVITRO APPROACH INVOLVING INFLAMMATORY TNF-A AND IL-6

Avishek Mandal[1], Rudranil Bhowmik[1], Md. Adil Shaharyar[1] Kumar Anand[1], Pallab Mandal[1], Jamal Akhtar [2], Samit Karmakar [3], Sanmoy Karmakar[1,*]

Abstract
This study was to investigate *invivo* and *invitro* hepatoprotective role of Indian earthworm (*Pheretima posthuma)* protein powder (EP) in rats against paracetamol induced liver damage. Briefly, the animals were divided into 4 groups (n=6) of wister albino male rats. Normal group, 1 ml of 20% Tween 80 (vehicle) was administered orally for 2 days in a week for 14 days. Paracetamol-treated group, paracetamol (750 mg/kg p.o.) suspension was administered orally twice a week for 14 days. Paracetamol-EP treated group, paracetamol suspension was administered concurrently with EP (266 mg/kg b.w), orally twice a week for 14 days. Paracetamol and silymarin treated group, paracetamol suspension was administered concurrently with silymarin (100 mg/kg p.o.) orally twice a week for 14 days. Paracetamol–treated animals exhibited a significant ($P<0.05$) elevation of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, LPO or MDA, total bilirubin, mRNA expressions of TNF- α and IL-6 while the level of CAT, SOD, GSH significantly ($P<0.05$) decreased in the paracetamol group and was later restored in paracetamol-EP treated group. Similarly, the separated MWCO 50 KDa protein obtained from EP (lyophilized) using Sephadex-G75 showed significant ($P<0.05$) reduction of AST and ALT, ALP and bilirubin in primary hepatocyte culture supernatant and increased percentage viability. The histological examination of paracetamol group showed disruption of the liver histoarchitecture and its restoration was found in the paracetamol-EP group. The *invivo* study, showed that the animals in the paracetamol-EP and the paracetamol-silymarin groups showed restoration of biochemical parameters, oxidative stress markers, mRNA expressions and histopathological findings. The acute liver damage due to paracetamol is attributed to the oxidative stress in the animal model. EP decreased oxidative stress as well as inflammation and slowed down the liver damage progression caused by paracetamol. EP (*Pheretima posthuma*) activities involving cytokine expressions e.g., TNF-α and IL-6 using lower molecular weight protein fraction from the dialysate i.e., MWCO 50 kDa and below protein fractions is of scientific importance with correlation to real time use.

Keywords: Paracetamol, Regeneration, Earthworm protein, Antioxidant and Hepatoprotective, SDS-PAGE.

[1*]Bioequivalence Study Centre, Dept. of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India.
[2]The Central Council for Research in Unani Medicine (CCRUM), Ministry of AYUSH, Government of India, Janakpuri, New Delhi-110058, India
[3]Medical College Kolkata, Kolkata, West Bengal, India

***Corresponding Author:**Prof. Sanmoy Karmakar
*Director, Bioequivalence Study Centre, Dept. of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India, sanmoykarmakar@gmail.com, +91-8017136385;

Co-authors: Avishek Mandal: avishek7477@gmail.com
Rudranil Bhowmik: rudranilz@yahoo.in
Md. Adil Shaharyar: adil503@yahoo.co.in
Kumar Anand: kr.anand.pharmamrit@gmail.com
Pallab Mandal: pallab.mandal3333@gmail.com
Jamal Akhtar: jamal7862@yahoo.co.in

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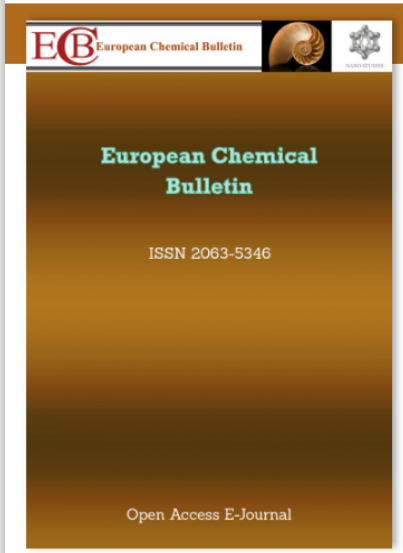

THE PROTECTIVE ACTION OF EARTHWORM PROTEIN POWDER/ MWCO 50 KDA FRACTION ON EXPERIMENTAL HEPATIC DAMAGE: AN INVIVO/INVITRO APPROACH INVOLVING INFLAMMATORY TNF-A AND IL-6

PDF

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Suggestions

Dear Author,

I am pleased to inform you that our reviewers and the section editor have recommended your manuscript for publication. Your manuscript has been scheduled for the first issue of Journal of Research in Pharmacy in January 2024. The proof of your manuscript will be provided in December, 2023. Thank you for choosing to publish in our journal.

Kindest regards

Pof.Hatice Kübra ELÇİOĞLU
Editor-in-Chief

Accepted

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Institutions:	[1]Jadavpur University, Department of pharmaceutical Technology, Kolkata, India [2]Jadavpur University, Bioequivalence Study Centre, Dept. of Pharmaceutical Technology, Kolkata, India
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SOCIETY FOR ETHNOPHARMACOLOGY, INDIA

(Globalizing Local Knowledge and Localizing Global Technologies)

Theme : Redefining Ethnopharmacology for the Global Health and Wellbeing

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. Avishek Mandal

has Presented a PAPER in ORAL/POSTER SESSION in the 9th International Congress of the Society for Ethnopharmacology, India (SFEC-2022) held at JSS College of Pharmacy, JSS Academy of Higher Education & Research, Mysuru, Karnataka, India from 22nd to 24th, April 2022.

Title of presentation: Ameliorative effect of the combination of Goat's Milk & Indian Earthworm extract on CCl4 Induced hepatotoxicity.

Dr. T.M. Pramod Kumar
Organizing Chairman, SFEC - 2022

Dr. K. Mruthunjaya
Organizing Secretary, SFEC - 2022

Mr. Birendra K. Sarkar
President - SFE - India

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Certificate of Participation

This is to certify that Dr./ Mr./Ms.

Avishek Mandal Research Scholar of *Jadavpur University, Dept. Of Pharmaceuticals Technology* has participated and presented his e-poster titled "*Goat's Milk's Potent Hepatoprotective*" in the two day webinar on 'Bioactive Glass, Ceramics & Composites in Healthcare: Current Technological Trends, 2021', held on 27th & 28th May, organized by Bio-Ceramic & Coating Division, CSIR-Central Glass and Ceramic Research Institute, Kolkata.

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This is to certify that Mr. Avishek Mandal, Research Scholar, of Department of Pharmaceutical Technology, Jadavpur University Kolkata has presented a research paper entitled ***Ameliorative effect of the combination of natural formulation Goat's milk and Indian Earthworm Extract powder on Ethanol- induced hepatotoxicity*** *in the International Conference on "Latest Trends on Applied Science, Management, Humanities and Information Technology" held at Sai College, Bhilai, Chhattisgarh, India on 26-27 May 2023.*

Dr. Mamta Singh
Convenor

Mr. H.S. Sachdev
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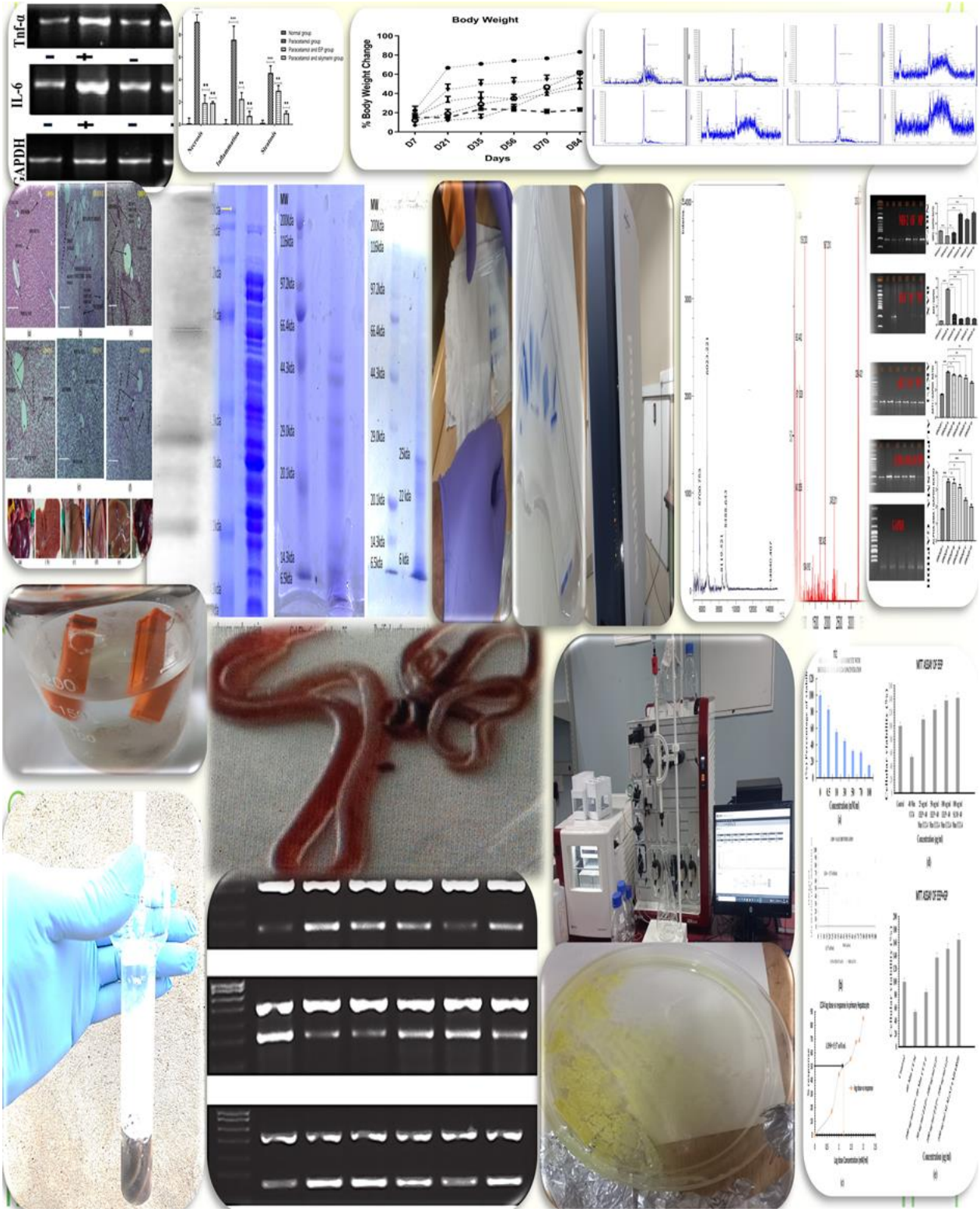
Dr. D.B. Tiwari
Principal

This Certificate can be verified at http://saicollege.org/conference/

EARTHWORM PROTEIN EXTRACTION PREPARATION AND HEPATOPROTECTIVE WORK OVERVIEW



EARTHWORM COLLECTION AND REARING