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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Abbreviation:
EP, Earthworm Protein powder; EE, Earthworm extract; B.W, Body Weight; SILYily, Silymarin; ALT, Alanine Transaminase; AST, Aspartate Aminotransferase; ALP, Alkaline phosphatase;TB, Total Bilirubin; LPO or MDA, Lipid Peroxidation or Malondialdehyde; ROS, reactive oxygen species; TNF-α, Tumour Necrosis Factor alpha ; IL-1, Interleukin 1; IL-6, Interleukin 6 ; IL-11, Interleukin 11; IL-10, Interleukin 10; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase,CAT, Catalase; GPx, Glutathione Peroxidase; GST,Glutathione (GSH) S-Transferase; SOD, Superoxide Dismutase ; Paracetamol, N-Acetyl-Para-Aminophenol Or Acetaminophen ; Kda,Kilodalton; NAPQI, N-Acetyl-P-Benzoquinone Imine; TCA, Trichloroacetic Acid; TBA, Thiobarbituric Acid; TBARS, Thiobarbituric Acid Reactive Substances; MTT,(3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; MCP, Monocyte chemoattractant protein; MIP-1, Macrophage inflammatory protein 1 ; MIP-1α, Macrophage inflammatory protein 1α; MIP-3a, Macrophage inflammatory protein 3a ;TIMP1, Tissue inhibitor of metalloproteinases ; αalpha-SMA, alphaα-Smooth muscle actin; BAX, Bcl-2 Associated X-protein; Bcl-2, B-cell lymphoma 2 ;VEGF, Vascular Endothelial Growth Factor; OTC, Over the counter; FDA, Food and Drug Administration; CYP, Cytochrome; mt-DNA, Mitochondrial DNA; JAK, Janus Activated Kinase; STAT, Signal Transducer And Activator Of Transcription; MAPK , Mitogen-Activated Protein Kinase; SDS PAGE, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; PCM, Paracetamol; APAP, Acetaminophen; KDa,Kilodalton; MWCO, Molecular weight cut-off; Mm, Milimolar, SOCS3, Suppressor of cytokine signaling 3.

1. 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].

2.MATERIALS AND METHODS

2.1.Material:

Indian Earthworm was purchased from Lila agrotech Pvt Ltd, Madhyamgram, Paracetamol (sigma-aldrich, catalogue no. A7085) and Silymarin was obtained (sigma-aldrich catalogue no. S0292), Aspartate aminotransferase (AST) kit (coral clinical system, catatalogue no 1102200025), Alanine aminotransferase (ALT) kit (coral clinical system, catalogue 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, catalogue number T22500), Minive vertical electrophoresis system (Amersham Biosciences corp. Amersham, United Kingdom), ImageQuant LAS 500 (GE Healthcare Life Sciences),Takara premixed protein marker (catalogue 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 catalogue no. 4368814), 1kb DNA Step Ladder (GCC biotech catalogue no. G4669), TAE Buffer 10 x (hi-Grade) (Thermo Fisher Scientific, India, catalogue number AM9869) and High gel agarose New, molecular biology grade (DNA/RNA) (GCC biotech, India. catalogue no. G4652).

2.2.Animals And Husbandry:

The animal studies were conducted according to the established methodology set by the Committee for the Monitoring and Control of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. All animal studies were carried out in accordance with the ARRIVE criteria and the National Institute of Health Guide for the Care and Use of Laboratory Animals. 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 were allowed to freely access food and water ad libitum. The room temperature was kept at 22±2 °C, with a 12-hour dark/light period.

2.3.Preparation of Indian earthworm protein powder:

200 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 total 40 mL of chloroform with methanol solution (1:1 ratio) and stored at 4 ℃ 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].

2.4.Animal Allocations and Treatment:

2.4.1.Hepatoprotective effect of EP in invivo experiment:

A Total of 24 albino Wister rats (male rat.150-180 g) were separated into 4 groups (n=6). The animals of normal group were administered 1 ml of 20% Tween 80 as 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.

2.4.2.Preparation of serum and tissue of the liver:

The rats were euthanized using pentobarbital (barbiturate) overdose, administered intraperitoneally, as per standard guidelines of 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 other part kept for PCR test with 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].

2.4.3. Activity of liver enzymes in the blood:

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

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, Glutathione (GSH) activity was measured using commercial kits.

2.4.5. Isolation of RNA:

From treated and untreated rats 100 mg of hepatic tissue samples were homogenized immediately after resection in guanidinium-based denaturation solution. Total RNA was isolated from the lysates using the RNA kit (Trizinxp RNA Isolation Kit) according to the manufacturer's instructions. DNase I was used to further 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 was used to evaluate TNF-α, IL-6 and GAPDH.

2.4.6. cDNA synthesis:

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

2.4.7.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, IL-6 is shown in the 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 usage of 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 second, 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 min 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 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.

2.4.8.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 was made in comparison 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 ana lysis 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 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, the mRNA expression of TNF-α and IL-6 were evaluated by end point agarose gel electrophoresis PCR. 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.

2.4.9. 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 was sectioned, deparaffinized, 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].

2.5.Isolation of primary hepatocytes:
Seglen's two-step perfusion in situ was an improvement on the inverse perfusion technique for obtaining primary hepatocytes from healthy rat. Prior to receiving paracetamol treatment, the hepatocytes were chosen and shown in a culture plate with 24 wells (5× 104 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 2 separate sets. The 1st set isolated primary hepatocytes was used for MTT assay and the 2nd set for AST, ALT, ALP, and total bilirubin.

2.5.1. Isolation of protein (protein fraction) from EP:
The crude earthworm was purified in different steps utilizing size exclusion chromatography (Sephadex G-75) and salting out using ammonium sulphate.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 sulphate 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 samples of protein 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].

2.5.2. Determination of cellular viability in MTT assay

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

2.5.3. Hepatoprotective effect of protein fraction in invitro 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 3 different doses of the concentrations of the earthworm protein MWCO 50 kDa , 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 for evaluating AST, ALT, ALP and total bilirubin [25].

2.5.4. 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 analysed using mini gel SDS PAGE. To prevent air from being trapped in the matrix, sephadex-G 75 beads were soaked in 50 mM phosphate buffer pH 6.0 for 48 hours while being slowly stirred. The completely swollen beads were put onto a (30 ×2 cm) polypropylene column. To prevent air entrapment and bed cracking, sephadex-G75 beads were packed under gravity with a repeated flow of phosphate buffer. To accomplish compact packing, the column was packed for 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, were 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 protein of filtrate 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 sephdex-G75 and MWCO 50KDa fraction.

2.5.5. Statistical analysis:

All the data were analysed 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.RESULTS:

2.6.Protein estimation:

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

3.2. EP attenuated liver toxicity markers induced by paracetamol:

Fig. 1a-1d and 7a-7d represents AST, ALT, ALP, and total bilirubin level respectively in both invivo and invitro study. 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 enzyme 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 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 paracetamol-silymarin treated group was more than paracetamol-EP treated group on 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.

3.3. EP attenuated oxidative stress markers induced by paracetamol:

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

3.4. 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 normal group (Fig.3).

In the paracetamol treated group, liver tissue sections stained with H&E reveal 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, as well as bile duct (BD) proliferation and hyperplasia, were both seen in the portal region.

Hepatic H&E-stained sections from the parace tamol-EP & paracetamol-silymarin treated groups revealed a remarkable improvement, as the majority 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). Figure 3b represents the necrosis, inflammation, and steatosis in terms of histopathological score. Necrosis, inflammation, and steatosis were found to be significantly ($p<0.001$) elevated in the paracetamol treated group. The three parameters were found to be significantly reduced ($p<0.01$) in paracetamol-EP and paracetamol-silymarin treated groups.

3.5.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 paracetamol group than in the normal group as indicated in Fig. 4: (a), (b), (c). The mRNA expression level of TNF-α and IL-6 were found to be significantly lowered ($P<0.05$) in the paracetamol-EP and paracetamol-silymarin groups in comparison to the paracetamol treated group. But it was observed that the reduction in the expressions of TNF-α and IL-6 in 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.

3.6. 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%, respectively (Fig 5).

3.7. 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 invitro viability assay indicates the % hepatocyte viability increases with increasing dose of earthworm MWCO 50 kDa.

3.8.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 level in the supernatant. As compared to the paracetamol induced disease control group (II), 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 (10 mg/ml) or group III and medium concentration earthworm protein MWCO (50 mg /ml) or group IV was found to decrease but non-significantly.

3.DISCUSSION:

The use of Earthworm finds its root in both Chinese and Indian traditional medicine system. The local (Dattapukur, West Bengal, India) use of this particular species (Pheretima posthuma) has also been prevalent for a long time. From this perspective, the earthworm used in 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 as a result of paracetamol overdose damage 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 (Figure 1a and 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 Figures 3a and 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 stabilisation 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, the accumulation of intracellular lipids leads to steatosis, a hallmark of liver disease [34]. Thereafter, the progression from steatosis to non-alcoholic steatosis hepatitis is influenced by a number of 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 and 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, in addition to other hepatoprotective effects, EP might lessen the degree of heme breakdown [37] and consequent biliary tract obstruction, finally 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 are amplified, mitochondrial membrane permeability transition pore opening, and ATP synthesis shutdown as a result of 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 both increased necrosis (Fig. 3a and 3b) and GSH depletion (Fig 1d) has been observed in our study in the paracetamol treated group.

Furthermore, following hepatic injury, replication of hepatic mitochondrial DNA (mt-DNA) 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 above mentioned mechanisms of action (s).

Apart from the explanations given above, another major aspect is that paracetamol induces lipid peroxidation, resulting in 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 in liver pathology, apoptosis and necrosis generally coexist, 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 to an increase in cyclin-D1 protein synthesis and liver regeneration in mice [40]. Together with IL-6, a multifunctional cytokine with both pathogenic and pro-inflammatory functions in autoimmune disease animal models [33], [42-43]. There are further reports claiming 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 with regards to 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 lead 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 culture 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 14kD Membrane). Thereafter, following different fractionation using spin column, the MWCO 50 Kda and lower molecular weight fraction(s) [29] was found to be more potent. Considering this particular approach with Pheretima posthuma, we feel this is much important in order to correlate observed in vivo and in vitro hepato protection of EP and its scientific justifications with real time usage.

3.CONCLUSION

Our report with EP against paracetamol-induced hepatocellular damage confirms with previous claims [14] involving reduction of oxidative stress and inflammation. Although, the genus and species of earthworm mentioned earlier was different to that 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.

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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.

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Competing Interests
The authors declare no competing interests.

Authors' Contributions
A.M. performed the experiment; S.K and S.K. designed the study; A.M., M.A.S and R.B. prepared the initial draft of the manuscript; A.M., J.A., P.M., K.A. revised the manuscript.

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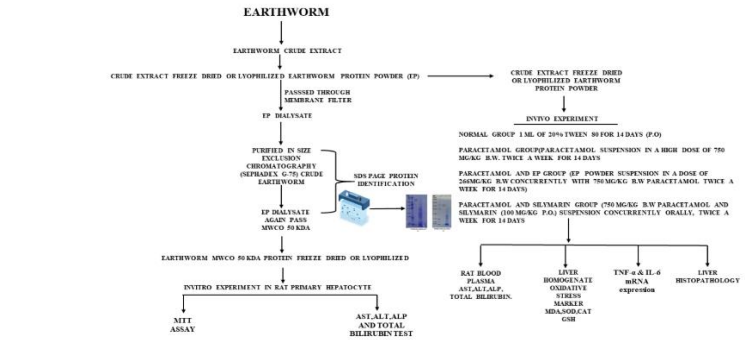
Figures:



Graphical abstract

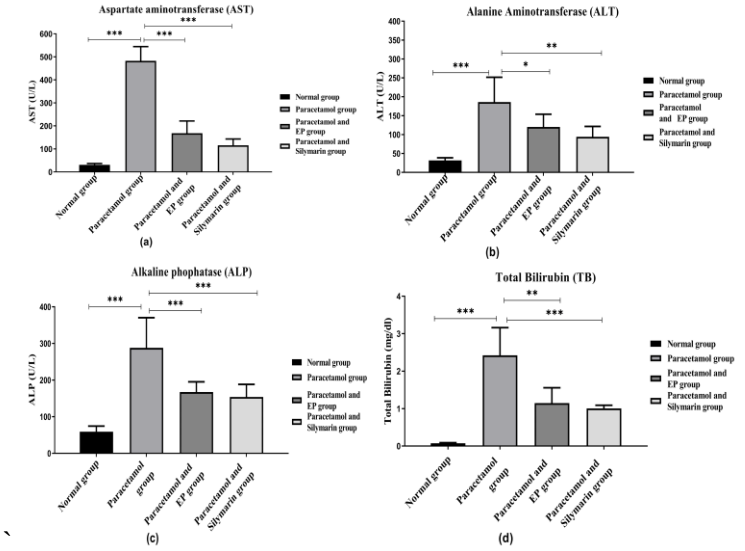


Figure 1: Effect of the EP (earthworm protein) on rat blood serum level (a) AST (U/L), (b) ALT (U/L), (c) ALP (U/L), and (d) Total bilirubin (mg/dl) concentrations were expressed respectively. Statistical comparison was made between normal group Vs paracetamol-treated group, paracetamol-treated group Vs paracetamol and EP protein- treated group, and paracetamol and silymarin (reference drug)-treated group and were found to be statically significant where *($p < 0.05$), **($p<0.01$) and ***($p<0.001$).

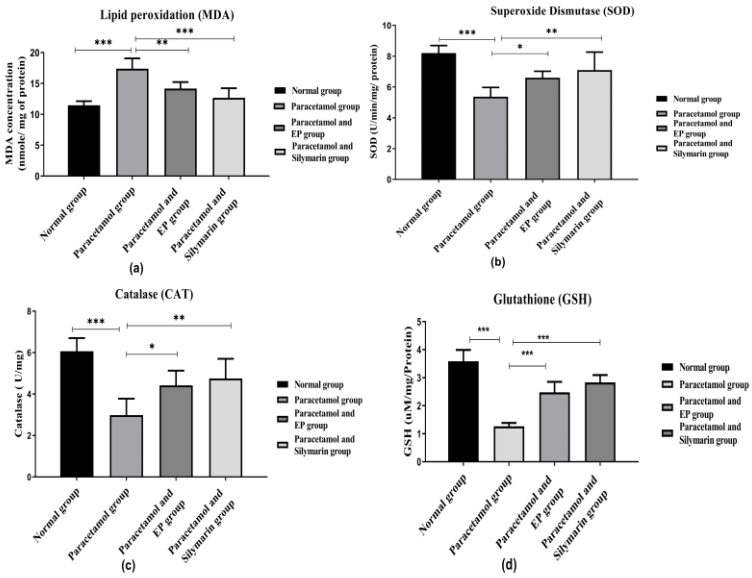


Figure 2: Effect of EP (earthworm protein) on liver tissue level of (a)MDA (b) SOD, (c) CAT (d) GSH. Statistical comparison was made between normal group Vs paracetamol-treated group, paracetamol-treated group Vs paracetamol and EP protein-treated group, and paracetamol and silymarin (reference drug)-treated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

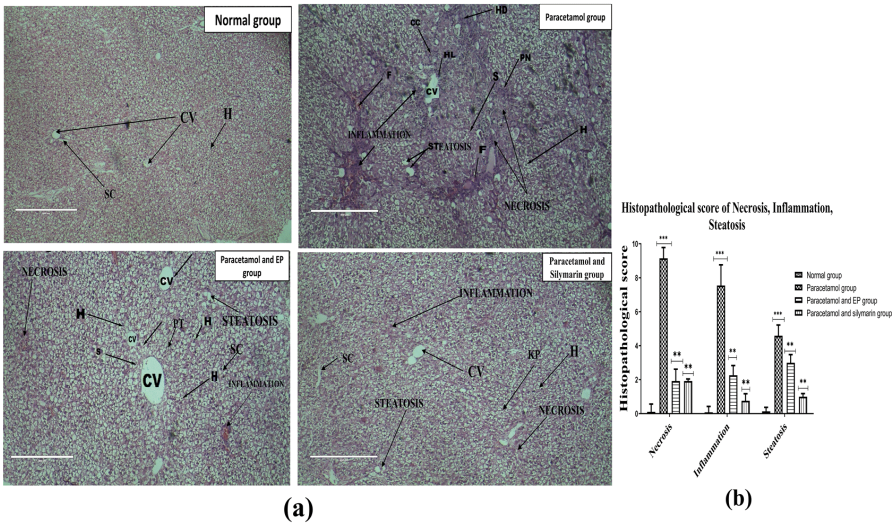


Figure 3: (a) Histopathological image of a Wistar rat liver (H&E, 10 X). Normal Group of the Wister rat liver sections was characterized by the presence of normal hepatic sinusoids (S), hepatic zonation, central vein (CV). No fibrosis development was noted and hepatocytes (H) were of appropriate shape. Paracetamol treated group showed Steatosis (ST) marked by arrow signs that indicate fatty changes (F), cell 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, as well as 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 in comparison to the normal group. No fibrosis occurring sign developed, no structural and central vein (CV) change in shape in paracetamol-EP treated group was found and showed restoration of the histoarchitecture. Paracetamol-Silymarin treated group showed more reduction in hepatic injury than the paracetaml-EP treated group. (b) Represents

histolopathological changes in terms of score taking into account Necrosis, inflammation and steatosis. Statistical comparison was made between 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).

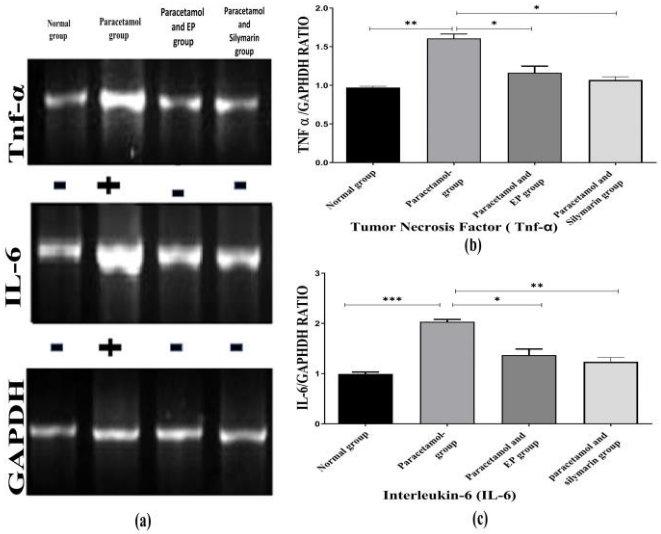


Figure 4: (a) Represents TNF-α, IL-6 and GAPDH gel doc Image (Image quaint LAS500 software densitometric analysis). The mRNA expressions of (b)TNF-α (c) IL-6 in all the 4 groups are also presented. Statistical comparison of the mRNA expression of TNF-α and IL-6 was performed between Normal group vs paracetamol 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 in (a) (+) and (-) signs indicates the expression intensity bands of TNF-α, IL-6 and GAPDH.

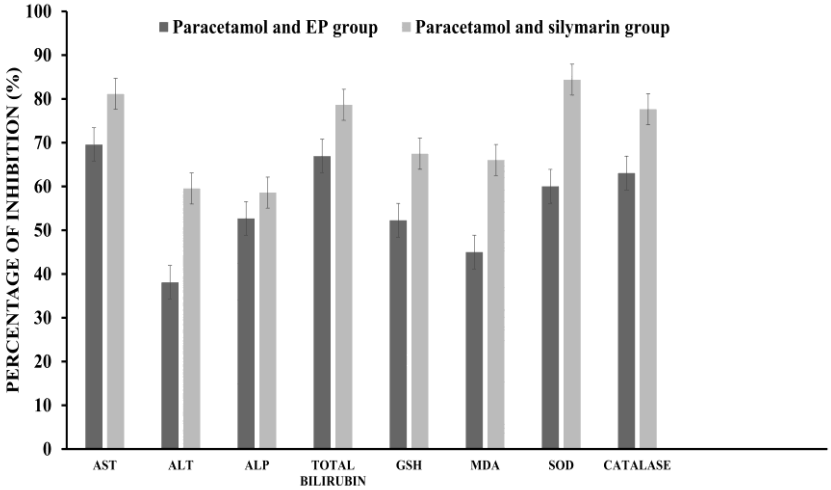


Figure 5: Represent the percent of inhibition of all the estimated liver biochemical parameters and oxidative stress markers in both paracetamol-EP treated and paracetamol-silymarin treated group in comparison to the paracetamol treated group which is considered as 100%.

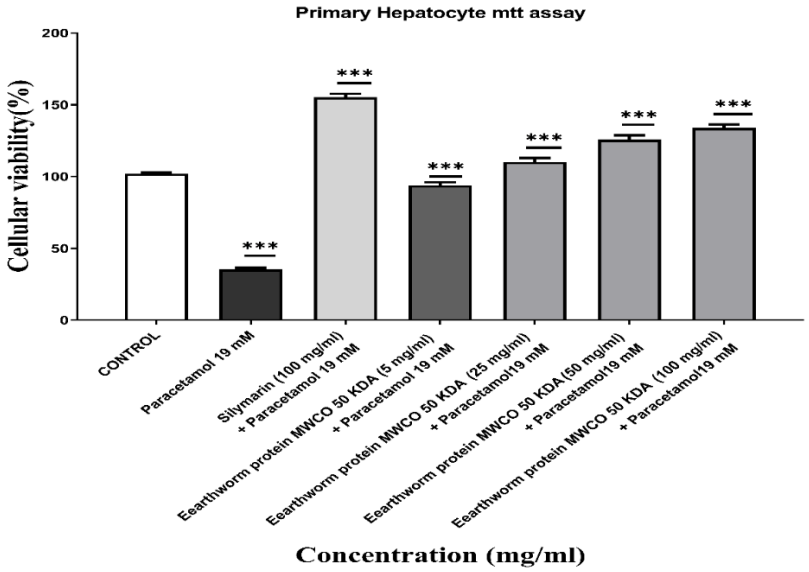


Figure 6: Cell viability of earthworm protein MWCO 50 kDa (dialysate) on rat primary hepatocyte. Rat primary hepatocytes were treated with Control (no treatment), paracetamol 19mM (disease control), (5-100) *m*g/ml earthworm protein MWCO 50 kDa with paracetamol 19 mM and Silymarin (SLM) (100 mg/ml+19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

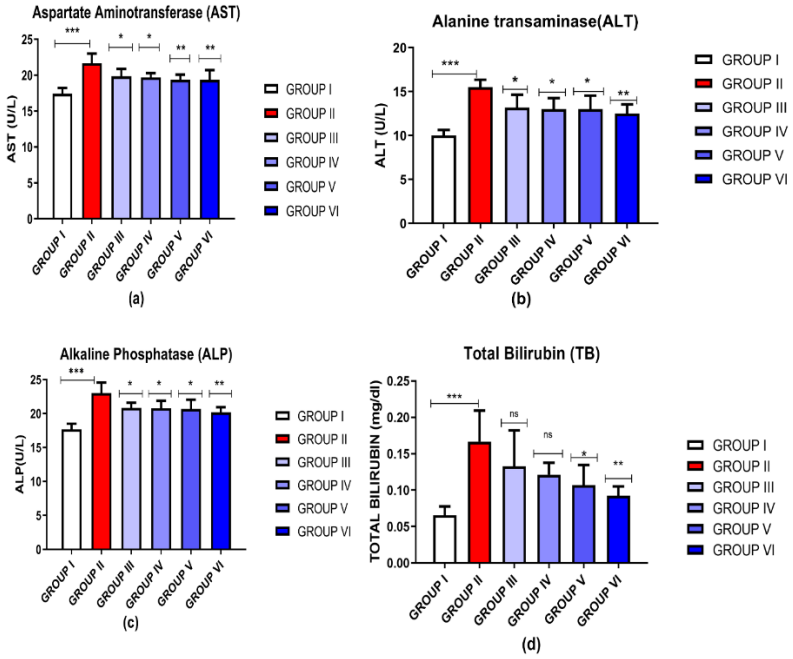


Figure 7: The changes of ALT, AST, ALP and Total bilirubin activities in primary hepatocyte culture supernatant. The control group or group I was compared with paracetamol induced disease control group or group (II) . The Paracetamol induced disease control group or group (II) was compared with the Low 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, *($p<0.05$), **($p<0.01$), ***($p< 0.001$).

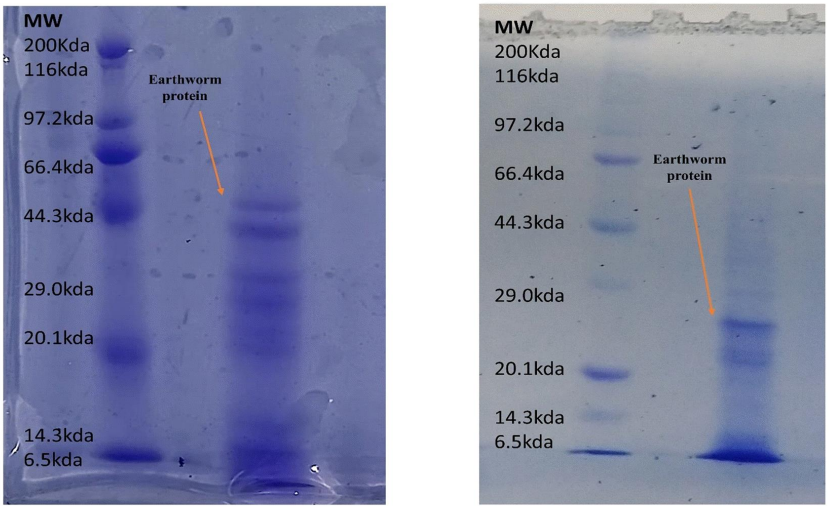


Figure 8: The molecular weight determination of purified earthworm protein from Indian earthworm. Fig 8(a) after gel filtration (Sephadex g-75) below 75 kDa and fig.8 (b) below MWCO 50 kDa protein band showed in SDS-PAGE.