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

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Received: 29 Mar 2023 / Revised: 12 July 2023 / Accepted: 15 July 2023

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), and lipid peroxidation or malondialdehyde (MDA) levels 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 ug/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 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 agent and prevent CCl4 treatment-induced primary hepatocyte toxicity.

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

1. INTRODUCTION

Large, small and complex various type proteins serve a variety of vital functions in the body. They are crucial for the structure, function, and control of the body's tissues and organs and carry out the majority of their job inside cells. Numerous thousands of amino acids, which are smaller building blocks of proteins, are linked together in lengthy chains to form proteins. Several bioactive enzyme and protein in earthworm have medicinal importance till now in research level [1]. 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 [2]. 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 [3]. As their pharmacological effects already found in anti-inflammation and antioxidative has enormous potential and potential for growth cellular mechanism. These findings may imply that the liver may be protected by the EEP. 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. It also has a higher content of short-chain fatty acids in milk fat,

How to cite this article: Karmakar S, Mandal A, Bhowmik R, Shaharyar A, Anand K, Mandal P, Sarkar A. Natural hepatoprotectives earthworm extract protein & goat milk *in-vitro* model rat primary hepatocytes exposed to carbon tetra chloride revealed toxicity and oxidative stress. J. Res. Pharm. 2024; 28(1): 258-268.

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http://dx.doi.org/10.29228/jrp.693

ISSN: 2630-6344

higher content of zinc, iron, and magnesium, and antibacterial characteristics [1]. 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 CCl4 [4]. Previous research shown that the livers of rats fed CCl4 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 [5]. 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 [6]. 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 [7]. CCl4 absorption from the gastrointestinal tract, it occurs quickly absorbed and is badly impacted in nutrition [8]. After ingestion, inhalation, or dermal absorption, CCl4 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 CCl4. But those people who worked in the chemical industry found inhalation experience to CCl4 has been found in several types of liver disease [7]. In addition, a study has already done oral, inhalation, and parenteral introduction to CCl4 has been found to develop hepatocellular carcinomas in animals [9]. 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 [3]. 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 [10]. Earthworm extract (EE) was revealed by Fu et al. to have the ability to stimulate osteoblast activation and cell proliferation in culture media [2]. Chang et al. studies that EE enhanced migration in Schwann cells by increasing the production of proteolytic enzymes that break down the matrix [2,3]. The pulmonary fibrosis and inflammation brought on by silica inhalation in mice may be significantly reduced by intraperitoneal injection of EE [11]. Several studies have reported hepatotoxicity in an in-vivo system of (0.5-2 ml/kg) body wt. in an animal model but have not found the exact toxicity starting level in CCl4 dose in in-vitro. Therefore, our study tries to determine the minimum dose of CCl4 in in-vitro primary rat hepatocytes where cellular cell viability is inhibited [12]. Earthworm extract mixed with goat milk is a traditional conventional therapy for liver illness used in INDIA, particularly by rural people in the West Bengal region of the Guma area. The current study aims to determine the lower to higher limit of dose CCl4 creates oxidative stress and causes cytotoxicity to the primary hepatocytes of rats. In our investigation, we created a model of EEP and EEP+GP induced primary hepatocytes in cultured liver cells, observed the protective effects, and investigated the underlying processes after 6 hours in CCl4-induced damaged hepatocytes.

2. RESULTS

2.1. Cell viability by MTT Assay

CCl4 showed a significant cytotoxic impact on primary hepatocytes. After 6 hours of exposure, the LD_{50} value was calculated (15.5 mM), where more than 50% of the cell were dead (Figure 2). At low concentrations (15.57 mM), CCl4 causes 50% loss in cell viability. At greater doses (0.5-100 mM), figure (1) showed a progressive decline in cell viability. Figure 1(d) & (e) showed in MTT assay EEP and EEP +GP on primary hepatocyte the cellular viability increased.

2.2. Lipid Peroxidation Assay

Assay for Lipid Peroxidation Figure (3) shows the impact of CCl4 on lipid peroxidation. The results of this experiment revealed that increasing concentration of CCl4 increased MDA generation in primary hepatocytes. In addition, the data in figure (3) showed that CCl4-induced toxicity increased MDA levels, an indicator of lipid peroxidation. These findings suggest that CCl4 causes LPO in response to oxidative stress

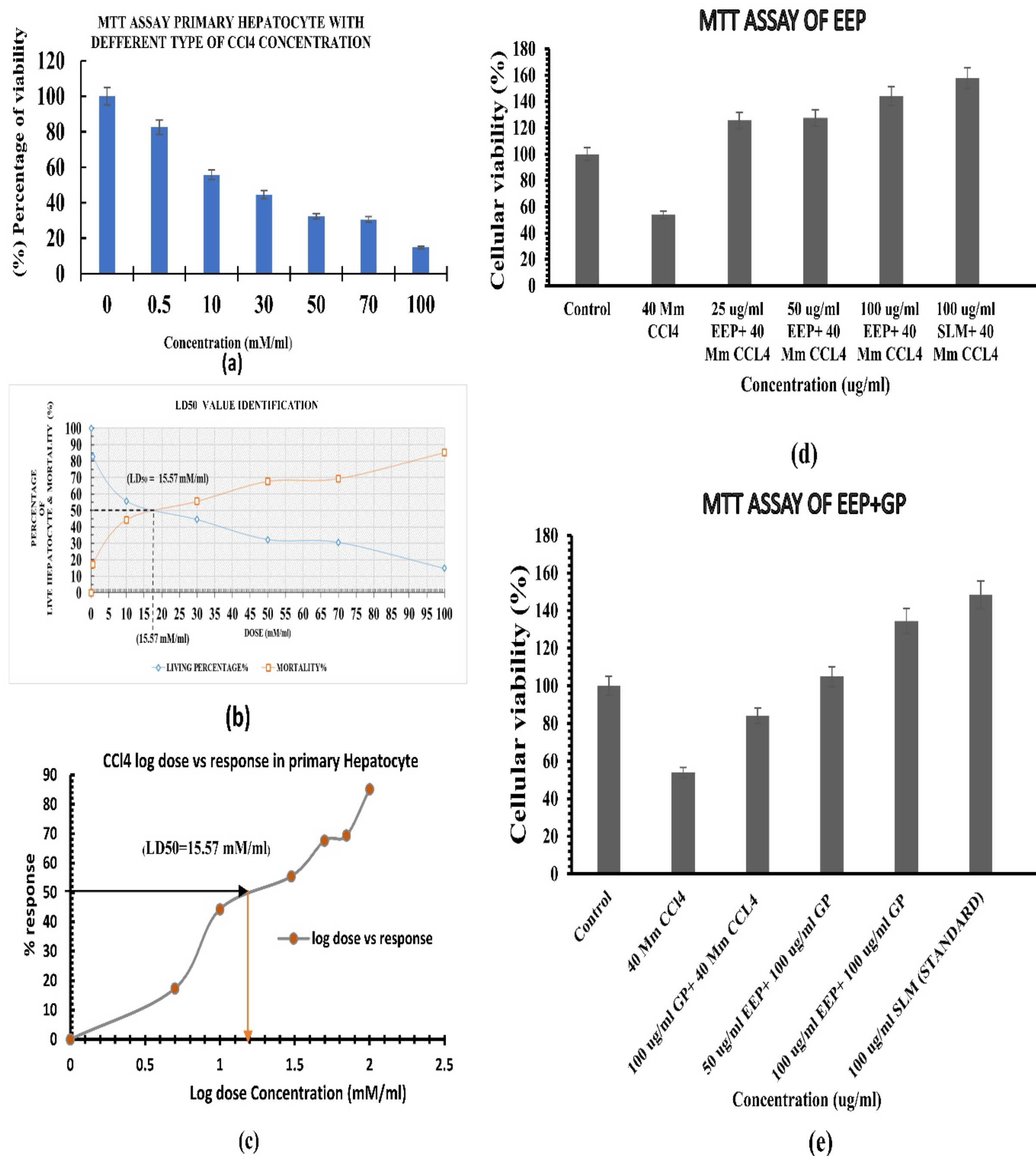

Figure 1. Percentage of cell viability in primary hepatocyte in a dose of 0.5,10,30,50,70 &100 (mM/ml) of CCl4 treatment showed data in excel plotted percentage of viability cell figure 1(a) decrease due to increase CCl4 dose. In figure 1(b) percentage of live hepatocyte vs mortality percentage plotted in excel data sheet for LD50 value identification. We got figure 1(b) &(c) LD50 value of 50% cells were died 15.57 mM/ml CCL4 treatment, here control sample value was showed figure 1(a)100% cell viability. But due to CCl4 toxic treatment, the cellular survival ability is lost due to dose increase. Here in figure 1 (d) & (e) we identified EEP and (EEP +GP) different concentration treatment in MTT assay.

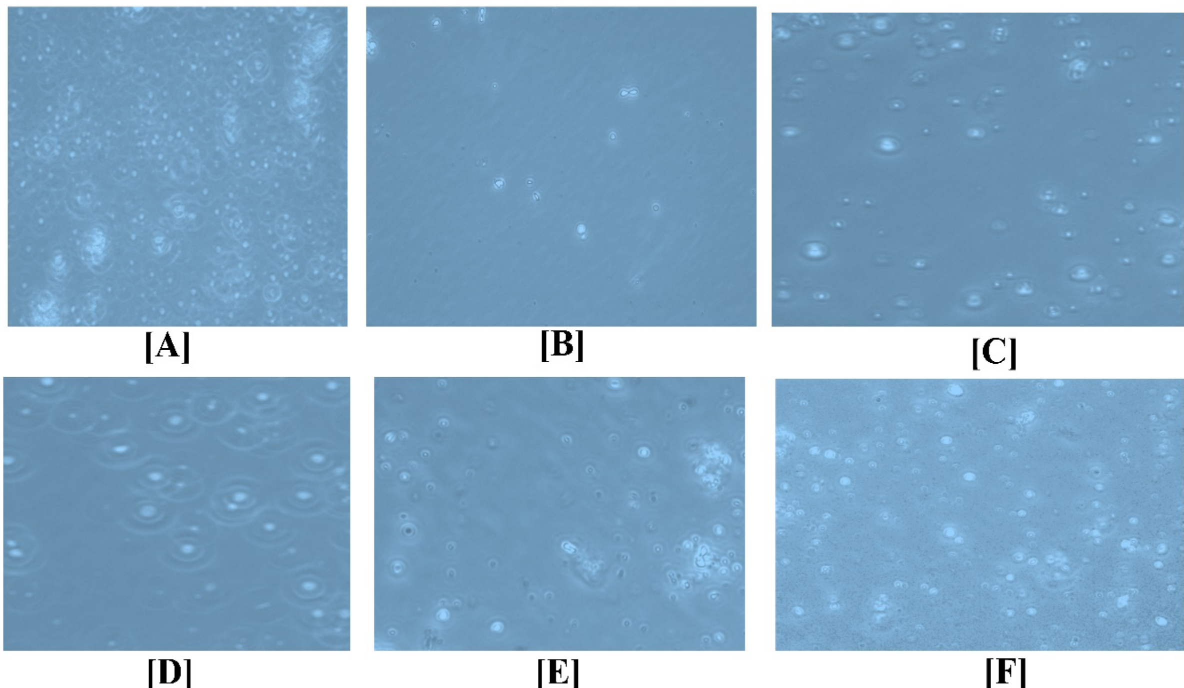

Figure 2. Isolated rat primary hepatocytes were observed under Carl Zeiss fluorescence Microscope, Magnification = 10x. After 6 hours, the primary hepatocyte control group's (no treatment) cellular viability was observed (A). the proliferation of hepatocyte decreases with the treatment of CCL4 dose 40 mM at figure2 [B]. Control figure 2 [A] were showed normal proliferation of primary hepatocyte, EEP treatment dose (25,50 & 100 ug/ml) [C], [D], [E] & Standard [F] (100 ug/ml) showed primary hepatocyte proliferation depending on their dose.

2.3. CCl4-induced cytotoxicity in Primary hepatocyte cells

The harmful effects of CCl4 depended on both time and concentration. Significant variations in cell viability, AST, ALT, lipid peroxidation, and tumor necrosis factor (Tnf-α) were seen when compared to the control figure3 & 4. up to a 6-hour exposure duration, these variations were still significant control vs CCl4 treatment vs treated test sample ($P < 0.05$).

2.4. CCL4-induced toxicity in primary hepatocyte culture and EEP protectant

The toxic effects of CCL4 are shown in Figure 3 & 4. The release of AST, ALT, LPO (or MDA level) and TNF-α increased during the 6 h in primary hepatocyte cells exposed to CCl4 and declined when EEP treatment without or with GP. The effect of CCl4 treatment with various concentrations showed a dose-dependent toxicity increase in primary hepatocyte cell culture. These changes remained significant up to an exposure time of 6 h ($P < 0.05$).

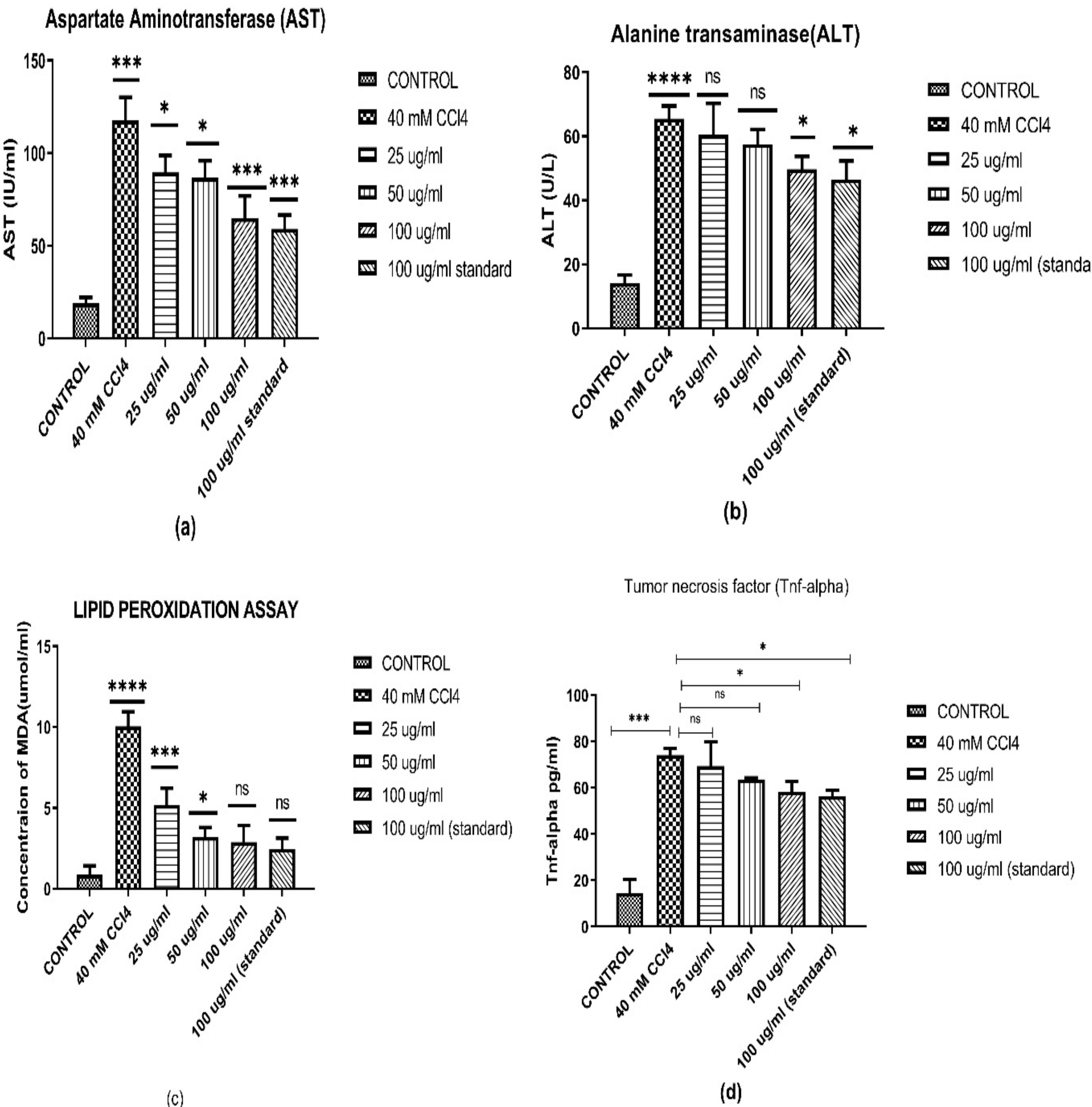

Figure 3. In figure.3 (a) & (b) EEP treatment at different dose were shown in our experiment both AST and ALT 100 ug/ml showed p- value were showed significantly changed **(p<0.01) & *(p<0.05). Very Low MDA levels can be seen in the control group. Various concentrations (25, 50 & 100 ug/ml) of EEP were evaluated for effect on the primary hepatocyte lipid in peroxidation assay. Here control vs 25 ug/ml and 50 ug/ml show significant p-value change ***(p<0.001) & *(p<0.05) but 100 ug/ml and 100 ug/ml (standard) treatment were not significantly changed 40 mM CCl4 treatment. Figure4(d). Tnf-α p-value were showed control vs CCl4 were significantly change ***(p<0.001) in figure4(d). In figure4(d). CCl4 vs EEP treatment with (25 and 50 ug/ml) were not showed Tnf-α significantly change p-value (p<0.05) but 100 ug/ml EEP showed p-value were significantly change *(p<0.05).

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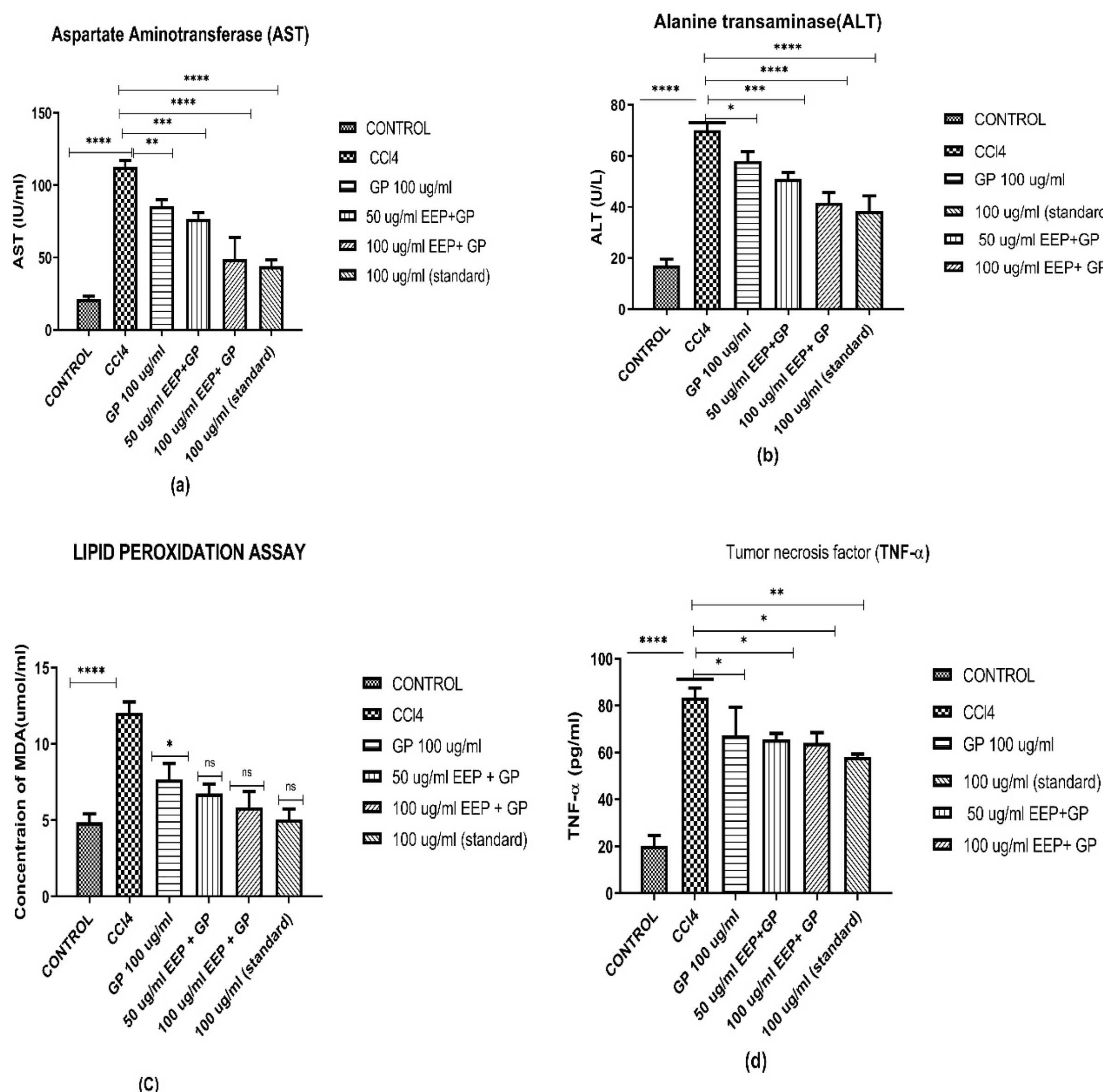

Figure 4. In figure 4 (a) & (b) EEP and GP treatment shown in our experiment both AST and ALT 50 and 100 (ug/ml) showed p-value were showed significantly changed ****(p<0.0001) & *(p<0.05). Very Low MDA levels increase seen in the control group. The concentrations (50 & 100 ug/ml) of EEP and GP were evaluated for effect on the primary hepatocyte lipid peroxidation assay. Here CCl4 group vs control groups treatment were shown significant p-value change ****(p<0.0001). But EPP and GP (50 & 100 ug/ml) treatment were not significantly changed MDA level at 40 mM CCl4 treatment. Figure 4(d). Tnf-α p-value were showed control vs CCl4 were significantly change ****(p<0.0001) in figure4(d). In figure4(d). CCl4 vs GP and EEP of Tnf-α concentration were showed significantly change p-value *(p<0.05) and **(p<0.01).

3. DISCUSSION

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 [18]. Multiple studies on novel medications and the efficacy of traditional medical practices have been published recently. The current study provides various types of disease treatment evidence for the conventional usage of protein derived from earthworm extract [19]. As a result, the extracts' ability to serve as antioxidants and as hepatoprotection against CCl4 liver-damaging effects were evaluated. In addition, numerous studies explained the association between liver damage and oxidative stress. The cytotoxic effect of CCl4 on primary hepatocytes was investigated in our experiment. Our findings reveal that CCl4 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 ug/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 ug/ml showed a positive hepatoprotective effect [16]. 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. In the current investigation, it was revealed that concomitant administration of goat milk and EEP together with CCl4 was helpful in lowering 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 natural products protein EEP can protect the liver. The mitochondria and impairing function of hepatic intracellular cells were evaluated for their hepatoprotective properties using the CCl4-induced liver toxicity technique. Because of its lipophilicity character, CCl4 spreads quickly across tissues and through cell membranes [4]. CCl4 induces cellular toxicity and inhibits cell proliferation. After 12 hours of culture, we found that CCl4 is cytotoxic to primary rat primary hepatocytes, with an LD_{50} of 15.57 mM/ml fig: 1(b). Our findings also show that initial doses of CCl4 (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, total bilirubin, and reduce total protein level in the blood plasma cause liver damage via oxidative stress in the animal model. Therefore, only the biomarker enzymes ALT and AST were examined in the current investigation. Compared to the standard medication silymarin, the present study in hepatoprotective activity results show that EEP have a higher amount of hepatoprotective activity. When compared to the toxic group (CCl4 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 increase. We used lipid peroxidation to investigate the probable role of MDA formation in CCl4 induced toxicity. The findings of this experiment revealed that CCl4 treatment significantly raises ($p<0.05$) MDA levels in primary hepatocyte culture media and also suggest that oxidative stress play an essential role in CCl4-induced toxicity. The reactive oxygen species (ROS) produced by CCl4 are well-known. 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 [20]. As previously observed in multiple study papers, CCl4 treatment caused acute liver damage that was strongly related with inflammation via increasing proinflammatory cytokine release [21]. TNF-α, a proinflammatory cytokine, was shown to be significantly increased in CCl4 treated generated primary hepatocyte culture in- vitro, representing a main cause of liver cell damage. It is remarkable that the administration of EEP and EEP+GP considerably decreased the release of these cytokines, indicating that it could play a function in reducing CCl4-induced liver damage. 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 [22]. According to In- vitro studies, CCl4 exposure produces superoxide, hydrogen peroxide, and hydroxyl radicals in epithelial cells [16]. 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 [23]. In our studies the hepatoprotective achievement involves primary hepatocyte growth in a culture medium and the restoration of hepatic MDA levels Figure3 & 4 with various type of treatment EEP and EEP+GP. The amelioration of CCl4-induced hepatotoxicity by EEP may also be caused by this mechanism. However, a more accurate application of this bioactive protective agent would depend on a better understanding of the underlying mechanisms behind EEP hepatoprotective effect.

4. CONCLUSION

The results of this investigation show that increasing dose exposure of CCl4 (0.5,10,30,50,70, & 100 mM/mL) significantly reduced the viability or increased cell cytotoxicity of the rat liver primary hepatocytes. Our study revealed the biological efficacy of EEP against cellular toxicity. However, the EEP proliferation effect was seen, as evidenced by a moderate increase in cell survival in primary hepatocytes compared to the other doses. At the molecular level, we found that CCl4 dose of 40 mM induces lipid peroxidation significantly increased ***(p<0.001). Eventually, EEP (25,50 & 100 ug/ml) and EEP+GP (50 & 100 ug/ml) revealed hepatoprotective activity after 6 hr of CCl4 40 mM/ml treatment. Future research needs to understand EEP mechanism of action and determine the right dose for the human usage to fully realize EEP promise as a protective agent. The findings from the current investigation may be effectively used to create appropriate clinical trials to assess only EEP or with EEP+GP effectiveness and safety in humans.

5. MATERIALS AND METHODS

5.1. Chemicals and cell culture media

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.

5.2. Lab safety

We employed high-efficiency particulate arrestor (HEPA) or charcoal filters in safety cabinets, lab hoods, and glove box rooms due to carcinogens invented by air. A commercially available filter enclosure is designed to allow the disposal of filters into plastic bags without contaminating maintenance personnel. After use, the plastic bag was promptly closed.

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

An earthworm breeding farm provided Indian earthworms (Pheretima 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 KD ultra-filter (Millipore) at 4 °C after the supernatant had been ultra-filtered with 50 KD ultra-filter (Millipore) with 5000 g for 20 min. Using Sephadex G-75, extract from the ultrafiltered solution was purified. Phosphate-buffered saline (PBS, 0.1 M, pH 7.8) was used to elute the desired protein sample, which was then kept at 20 °C. The sample was then centrifuged for 30 minutes at 1200 g in 4 °C. In order to extract the remaining crude protein, the pellets were dissolved in cold acetone and recentrifuged. The crude protein was lyophilized, then powder form protein kept at 20 °C until used. The marketed lyophilized goat milk (GP) were used. EEP and EEP+GP both lyophilized powders were used with DMEMA in DMSO (0.05%).

5.4. 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 CCl4 for 6 h. For EEP 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 6 h. For SLM treatment, cells were incubated with DMEMA with SLM100 μg/mL for 12 h and then treated with 40 mM CCl4 for 6 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 maintain 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 3×104 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) was 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 [13,14]. Each assay was done in triplicate.

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

The cell viability was determined using MTT assay. This colorimetric test uses mitochondrial succinate dehydrogenase, only found in living cells, to transform MTT into a purple formazan colour. The primary hepatocyte culture were treated EEP and EEP+GP with different concentration. 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 [15].

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

As evaluated by lipid peroxidation levels in rat primary hepatocytes, CCl4 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 [16]. 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 [17]. 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.

5.7.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, GM and the toxic agent CCl4. 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 value were measurement according to the supplied commercial kit.

5.8.CCl4 cell viability assay

In vitro assay to identify hepatotoxicity effects of different doses of CCl4. The hepatotoxic effects of CCl4 treatment 0.5,10,30,50,70 and 100 mM on primary hepatocyte cells were measured. CCl4 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 CCl4, we evaluated the LD50 value was calculated (15.57 mM), where more than 50% of the cell were dead.

5.9. Statistical Analysis

All data were given as means, ± SD (standard deviations). For each treatment sample, the triplicate test was performed, and statistical analysis was performed using one-way analysis of variance (ANOVA) for lipid peroxidation assay. Statistical significance was considered for ($p<0.05$). Using the Microsoft Excel application, the percent (%) of cell viability, LD50 value. The GraphPad prism 9 from the Tukey test graphically represented AST, ALT, TNF-α and MDA levels.

Acknowledgements: We gratefully appreciated the financial assistance provided by the UGC (Rajiv Gandhi National Fellowship, F1¬17.1/2014¬15/RGNF¬2014¬15¬SC¬WES¬78683 /(SA¬III/Website) in India. 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.

Compliance with ethical standards: 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.

Author contributions: Concept – S.K., A.M; Design – S.K., A.M., R.B.; Supervision – S.K.; Resources – S.K., A.M.; Materials – A.S., P.M; Data Collection and/or Processing – A.M., R.B., A.S., M.A.S.; Analysis and/or Interpretation – A.M., M.A.S, K.M., P.M., A.S.; Literature Search – M.A.S., K.A., A.S.; Writing – A.M., K.A., R.B., M.A.S.; Critical Reviews – A.M., M.A.Ş., A.S., P.M., K.A.

Conflict of interest statement: The authors declared no conflict of interest.

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