Original Article

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Hepatoprotective properties of the glycolipoprotein extract from *Eisenia foetida*

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Abstract

The liver is continuously exposed to a variety of xenobiotics. Several xenobiotics are identified as hepatotoxicants. Hence, finding protective agents for ameliorating xenobiotics-induced liver injury has a great value. *Eisenia foetida*, a kind of "earthworm," is a source of a wide range of bioactive components. Several investigations have been evaluated the *E. foetida* extract (EFX) for biomedical and nutritional applications. The current study was designed to evaluate the potential hepatoprotective properties of EFX in two experimental models of hepatic damage. Acetaminophen (APAP; 1 g/kg, i.p) was administered as the animal model of acute liver injury in mice. Bile duct ligated (BDL) rats were used as the animal model of chronic hepatic damage. Severe elevation in tissue biomarkers of oxidative stress including lipid peroxidation and hepatic glutathione depletion was evident in both APAP-treated and BDL animals. Moreover, serum biomarkers of liver injury were drastically increased in both acute and chronic animal models of hepatotoxicity. Significant liver tissue histopathological alterations including tissue necrosis, vascular congestion, and inflammatory cells infiltration were detected in APAP-treated and BDL animals. On the other hand, it was found that EFX supplementation (100, 200, 500, and 700 mg/kg, i.p) mitigated oxidative stress markers, decreased serum biomarkers of liver injury, and alleviated liver tissue histopathological changes. The hepatoprotection provided by EFX supplementation in the current study might be mediated through its potential antioxidative mechanisms.

Keywords: Antioxidant, Hepatotoxicity, Liver injury, Natural products, Regenerative medicine.

1. Introduction

Liver is continuously exposed to a wide range of xenobiotics. Exposure to xenobiotics or their reactive metabolites might lead to liver injury (1-3). Therefore, finding hepatoprotective agents has a great value. Recently a great interest has been emerged to find biologically active hepatoprotective agents from natural resources. In this context, a wide range of herbal medicines and their active components have been tested in different experimental models of liver injury (4-10). Amino acids, peptides, proteins, and polyphenol chemicals as well as many other compounds of natural origin are widely evaluated for their protective properties, over and above their potential in regenerating the damaged tissues (11-25).

Earthworms have been used for their great

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therapeutic advantages in different regions of the world since ancient times (26, 27). It has been reported that earthworm contains a wide range of bioactive components with different activities (28-32). Interestingly, many therapeutic properties are attributed to earthworm preparations. Antimicrobial, anti-inflammatory, antioxidant, antitumor, and wound healing properties of earth worm-based preparations were documented in previous studies (33-35). On the other hand, it was reported that earthworm extract provided protective properties against xenobiotics-induced organ injury (36).

The current study attempted to investigate the potential hepatoprotective role of the glycoprotein extract from the earthworm *Eisenia foetida* in two experimental models of liver injury.

2. Materials and methods

2.1. Chemicals

Hydroxymethyl aminomethane hydrochloride (Tris-HCl), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), methanol, and n-butanol were purchased from Merck (Darmstadt, Germany). Thiobarbituric acid (TBA), potassium chloride (KCl), 5,5'-dithiobis-(2-nitrobenzoic acid; DTNB), and phosphoric acid were obtained from Sigma chemical company (Sigma-Aldrich, St. Louis, MO). All other chemicals for preparing buffer solutions were of the highest grade commercially available.

2.2. E. foetida extract preparation

Earthworms, *E. foetida* were purchased from the stock culture, Alborzparseh ®Company (Shiraz, Iran). Earthworm extract was prepared according to the chloroform/methanol extraction method as previously described (37) (Figure 1). Briefly, worms were kept at 0.65% NaCl solution at room temperature for 2 h until their digestive systems became clean. Meanwhile, the solution was changed three times (Figure 1). Earthworms were kept out of the solution and minced with scissors. Then, three grams of earthworms was homogenized in 40 ml of chloroform/methanol (v/v) solution and kept overnight at 4 °C. Subsequently, 16 ml of distilled water was added to the homogenate and samples were vortexed (Figure 1). The mixture was centrifuged (10,000 g, 4 °C, 10 min) to obtain three visible layers. The upper, water/methanol layer was pipetted out, and the methanol content was removed using a rotary evaporator. The final opalescent fluid was freeze-dried and stored at 4 °C (37) (Figure 1).

2.3. Animals

Male Sprague-Dawley rats (200-250 g) and BALB/c mice (Male, 20-30 g) were purchased from the Comparative and Experimental Medicine Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. Animals were acclimated to an environmentally controlled room (24±1 ºC, 45%±5% relative humidity, and a 12 h light/ dark cycle). Animals had free access to a rodents commercial pellet diet (Behparvar®, Tehran, Iran) and tap water ad libitum. The animal experiments were conducted by the guidelines for the care and use of

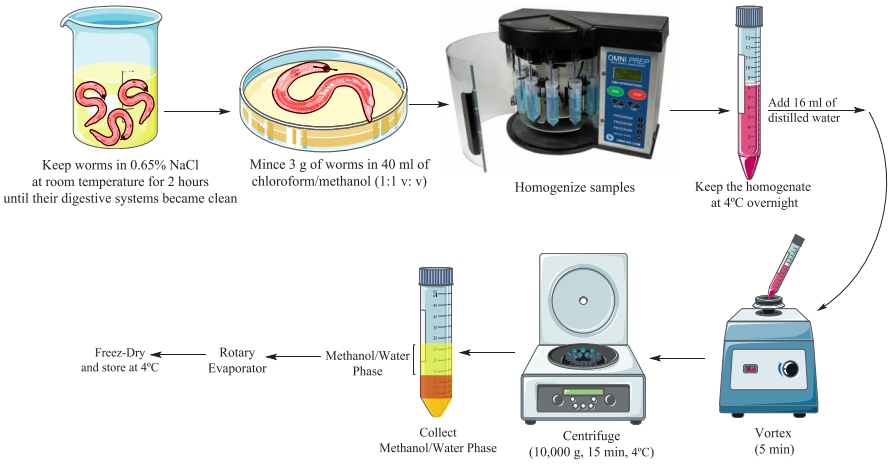

Figure 1. Schematic representation for preparing the glycoprotein extract from the earthworm *Eisenia foetida*.

laboratory animals approved by the institutional animal care and use ethics committee of Shiraz University of Medical Sciences (6488/6489).

2.4. The animal model of acute liver injury

Acetaminophen (paracetamol; APAP) is a regularly used drug to induce hepatic damage in experimental animals (38-40). Mice are more susceptible to acetaminophen hepatotoxicity than rats (41). In the current investigation, a high dose of acetaminophen (1 g/kg, i.p) was administered to mice, and liver injury biomarkers were assessed 24 h after drug administration (42). EFX (500 and 700 mg/kg, i.p) was administered two h after acetaminophen. Control animals received normal saline (NaCl 0.9% w: v, 2.5 ml/kg, i.p).

2.5. Bile duct ligated (BDL) rats

Animals were anesthetized (10 mg/kg of xylazine and 70 mg/kg of ketamine, i.p), a midline incision was made and the common bile duct was localized, doubly ligated, and cut between these two ligatures (43-46). The sham operation consisted of laparotomy and bile duct identification and manipulation without ligation. Animals were equally allotted into four groups (n=6/group) including: 1) Sham-operated (vehicle-treated); 2) BDL; 3) BDL + EFX (100 mg/kg/day, i.p, started from day 1 after BDL operation); 4) BDL + EFX (200 mg/kg/day, i.p, started from day 1 after BDL operation), and 5) BDL + Silymarin (200 mg/kg/day, i.p, started from day 1 after BDL operation). Liver and blood samples were collected 14 days after BDL operation.

2.6. Liver glutathione content

The hepatic glutathione (GSH) content was determined by assessing non-protein sulphydryl contents with the Ellman reagent (47, 48). Briefly, liver samples (200 mg) were homogenized in 8 ml of ice-cooled EDTA solution (20 mM, 4 ºC). Then, 5 mL of homogenized tissue was mixed with 4 mL of distilled water and 1 mL of trichloroacetic acid (TCA; 50 % w/v, 4 ºC). Samples were mixed and centrifuged (15 min, 10,000 g, 4 ºC). Afterward, 2 mL of the supernatant was treated with 100 µl of the Ellman reagent (DTNB, 0.01M in pure methanol) (48-51), and the absorbance of the developed color was measured at λ=412 nm using an EPOCH plate reader (BioTek®, Highland Park, USA).

2.7. Determination of lipid peroxidation in the liver tissue

Thiobarbituric acid reactive substances (TBARs) were measured as an index of liver lipid peroxidation in the liver tissue. Briefly, 500 mg of the liver tissue was gently minced and homogenized in 5 ml of ice-cooled (4 ºC) KCl solution (1.15% w: v) (47, 52, 53). Afterward, .05 mL of the tissue homogenate was treated with 3.5 ml of a buffer containing 0.75 mL of thiobarbituric acid (0.8%, w:v), 0.75 mL of 20% phosphoric acid (pH=3.5) and 0.1 mL of sodium dodecyl sulfate (8.1%, w:v) (48-52, 54). Samples were heated in a water bath (100 ºC, 45 min) (47, 52, 53). Then, 2 mL of n-butanol was added and vortexed (5 min). Samples were centrifuged (10,000 g for 5 min). The absorbance of developed color in the n-butanol (upper phase) was measured at λ=532 nm using an EPOCH plate reader (BioTek®, Highland Park, USA) (47, 52).

2.8. Blood biochemistry

Blood samples were collected from the abdominal vena cava. Samples were transferred to EDTA-coated or gel and clot activator containing tubes and centrifuged (10,000 g, 5 min, 4 ºC) to prepare blood plasma and serum. A Mindray BS-200® autoanalyzer and standard commercial kits (Pars Azmoon®, Tehran, Iran) were used to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (55, 56).

2.9. Histopathological studies

Liver samples were fixed in 10% phosphate-buffered formalin (0.4% sodium phosphate monobasic, NaH_2PO_4, 0.64% sodium phosphate dibasic, Na_2HPO_4, pH=7.4) for at least 24 h. Then, the paraffin-embedded sections were prepared and cut into 5 µm thick sections in a rotary microtome. Tissue sections were stained with the hematoxylin-eosin dye (H&E) and evaluated for histopathological alterations blindly (57).

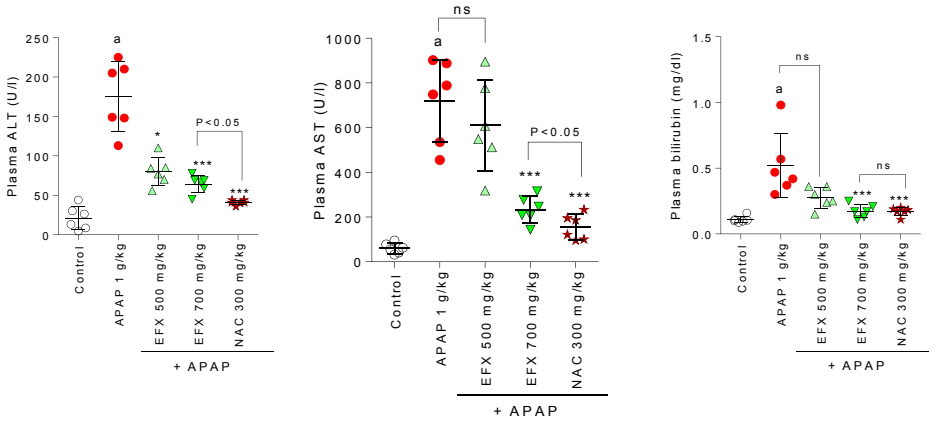

Figure 2. Plasma biomarkers of liver injury in acetaminophen (APAP)-treated animals. EFX: *Eisenia foetida* extract; NAC: N-acetylcysteine.Data are expressed as Mean±SD (n=6).
[a]Indicates significantly higher than control (Sham-operated) group (P<0.001).
***Indicates a significant difference as compared to the APAP group (P<0.001).
ns:not significant.

2.10. Statistical analysis

Data are given as Mean±SD. Comparison of data sets was performed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparisons as the post hoc test. Values of P<0.05 were considered statistically significant.

3. Results

Serum biomarkers of liver injury were drastically elevated in APAP-treated mice in comparison to control animals (Figure 2). Significant elevation in plasma ALT, AST, and total bilirubin were detected in the APAP group (Figure 2). On the other hand, it was found that EFX supplementation (500 and 700 mg/kg, i.p) decreased plasma level of liver injury biomarkers in APAP-treated animals (Figure 2). NAC administration as a standard treatment of APAP hepatotoxicity also significantly alleviated the increase in plasma biomarkers of hepatotoxicity (Figure 2).

It was found that liver tissue markers of oxidative stress were deteriorated in the APAP

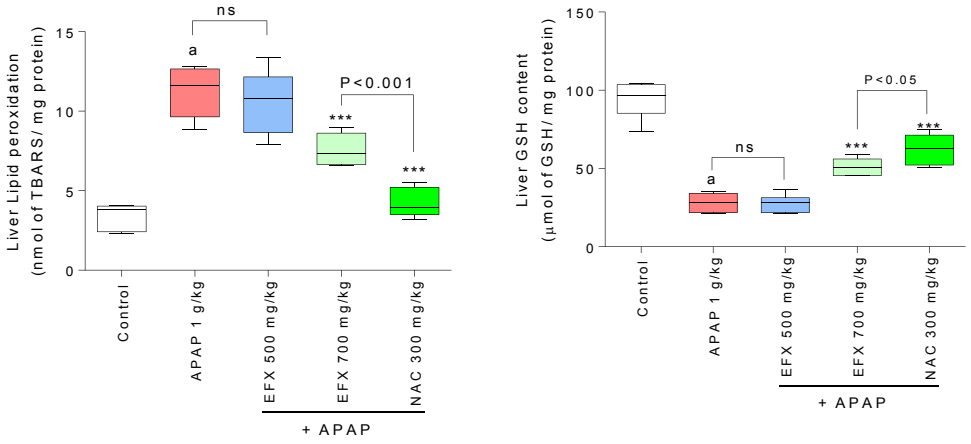

Figure 3. Markers of oxidative stress in the liver tissue of acetaminophen (APAP)-treated mice and the role of *E. foetida* extract (EFX) supplementation. NAC: N-acetylcysteine.
Data are expressed as Mean±SD (n=6).
[a]Indicates significantly higher than control (Sham-operated) group (P<0.001).
***Indicates a significant difference as compared to APAP group (P<0.001).
ns: no statistically significant difference.

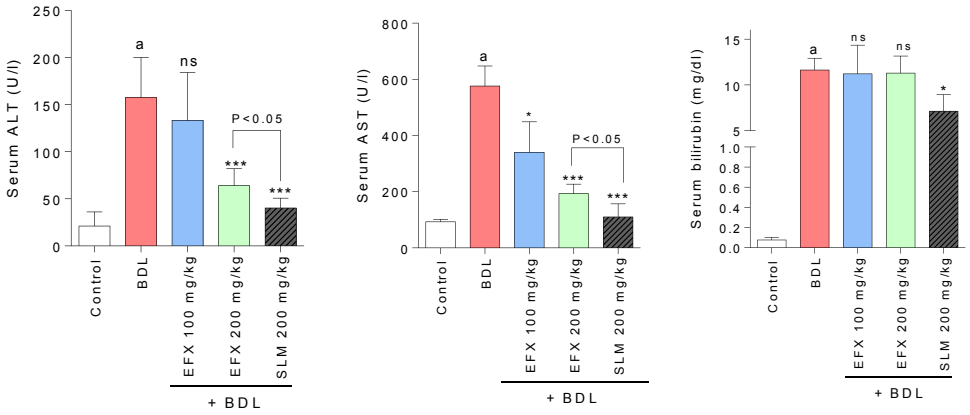

Figure 4. Serum biomarkers of liver injury in bile duct ligated (BDL) rats. EFX: *E. foetida* extract; SLM: Silymarin. Data are expressed as Mean±SD (n=6).
[a]Indicates significantly higher than the control group (P<0.001).
***Indicates a significant difference as compared to BDL group (P<0.001).
ns: not significant as compared to the BDL group.

group (Figure 3). A significant increase in lipid peroxidation along with hepatic tissue glutathione depletion was evident in APAP-treated mice (Figure 3). It was found that EFX administration (500 and 700 mg/kg, i.p) mitigated APAP-induced oxidative stress in the liver tissue (Figure 3). NAC (300 mg/kg, i.p) was also able to abrogate APAP-associated oxidative stress in mice liver tissue (Figure 3).

Serum biomarkers of liver injury were significantly increased in BDL rats in comparison to control animals (Figure 4). On the other hand, it was found that EFX supplementation (100 and 200 mg/kg, i.p for 14 consecutive days) decreased serum level of liver injury biomarkers in BDL rats (Figure 4). Administration of silymarin (200 mg/kg) also provided hepatoprotective properties in BDL animals (Figure 4).

Liver tissue markers of oxidative stress were significantly changed in BDL animals compared with the control group (Figure 5). A significant level of lipid peroxidation along with depletion of hepatic glutathione stores was evident in BDL animals (Figure 5). It was found that EFX

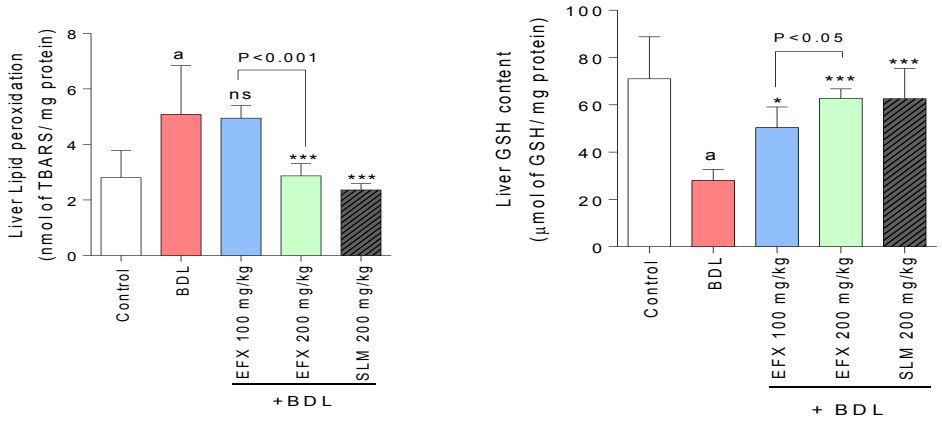

Figure 5. Effect of *E. foetida* extract (EFX) on liver oxidative stress markers in bile duct ligated (BDL) rats. SLM: Silymarin.
Data are expressed as Mean±SD (n=6).
[a]Indicates significantly higher than control (Sham-operated) group (P<0.001).
***Indicates a significant difference as compared to BDL group (P<0.001).
ns: not significant as compared to the BDL group.

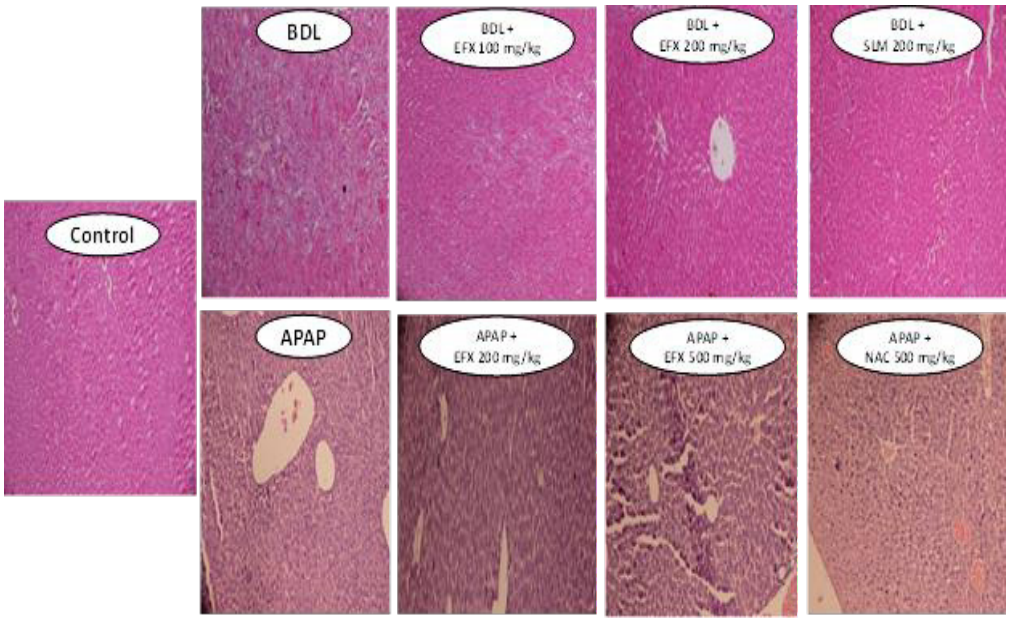

Figure 6. Liver histopathological changes in acetaminophen (APAP)-treated mice and bile duct ligated (BDL) rats. The grades of liver tissue histopathological alterations are shown in Table 1. EFX: *E. foetidae* extract; SLM: Silymarin; NAC: N-acetyl cysteine.

administration (100 and 200 mg/kg, i.p for 14 consecutive days) alleviated BDL-associated oxidative stress in the liver tissue (Figure 5). Silymarin treatment (200 mg/kg, i.p for 14 consecutive days) also mitigated biomarkers of oxidative stress in the liver of BDL animals (Figure 5).

Significant liver tissue histopathological alterations, including vascular congestion, inflammatory cells infiltration, and tissue necrosis, were evident in APAP-treated mice in comparison to control animals (Figure 6 and Table 1). On the other hand, it was found that EFX (500 and 700 mg/kg, i.p) or NAC (300 mg/kg, i.p) alleviated APAP-induced liver tissue histopathological alterations (Figure 6 and Table 1). Liver tissue necrosis, in-

Table 1. Hepatic tissue histopathological changes in the acute and chronic experimental models of liver injury.

Treatments	Congestion	Necrosis	Inflammation
Control	-	-	-
APAP	++	+++	+++
+ EFX 500 mg/kg	++	++	++
+ EFX 700 mg/kg	++	+	++
+ NAC 300 mg/kg	+	+	+
BDL	+++	+++	+++
+ EFX 100 mg/kg	++	++	++
+ EFX 100 mg/kg	++	++	++
+ SLM 200 mg/kg	++	+	+

$^{+}$:Mild;

$^{++}$: Moderate;

$^{+++}$:Severe tissue histopathological alterations.

EFX: *Eisenia foetida* extract;

NAC: N-acetylcysteine;

SLM: Silymarin.

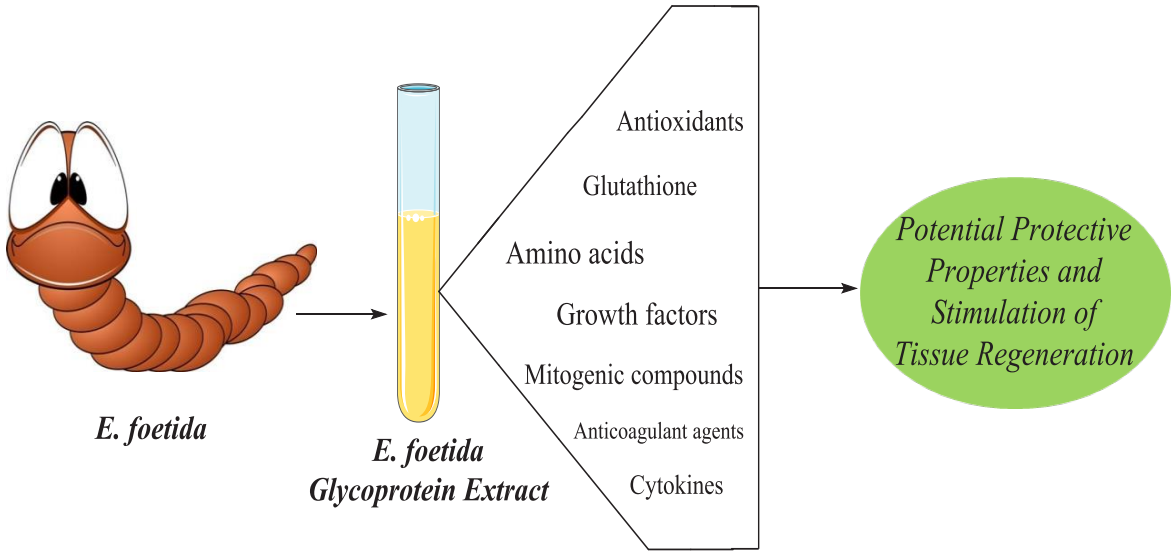

Figure 7. A diagrammatic view of the potential bioactive components of earthworm extract from *Eisenia foetida* and their role in alleviating xenobiotics-induced tissue injury and the stimulation of tissue regeneration.

flammation, and vascular congestion were also evident in BDL rats (Figure 6 and Table 1). EFX supplementation (100 and 200 mg/kg, i.p for 14 consecutive days) or SLM (200 mg/kg) decreased BDL-associated liver histopathological alterations (Figure 6 and Table 1).

4. Discussion

Liver injury occasionally occurs upon exposure to different xenobiotics. The current study was designed to evaluate the hepatoprotective properties of the glycoprotein extract from *E. foetidae*. It was found that the extract supplementation alleviated blood biomarkers of liver injury and mitigated liver tissue oxidative stress in both acute and chronic animal models of hepatotoxicity.

A focus has been emerged to find new and safe molecules with therapeutic capability against different diseases. Due to its strategic role in the detoxification of xenobiotics, liver tissue is continuously exposed to a high level of foreign compounds or their reactive metabolites. Hence, finding molecules to protect the liver, boosting its regeneration capability, and enhancing its defense mechanisms has a great value.

Earthworms are widely consumed in different regions of the world as a food source or in folk medicine (58, 59). *E. foetidae* is a kind of earthworm investigated for its therapeutic value. Several biologically active compounds have been isolated from *E. foetidae* (60-63). *E. foetida* extract has been studied for its protective properties against different xenobiotics-induced organ injury (31, 64). Several pharmacological activities including antioxidant, anti-inflammatory, and mitogenic actions have been attributed to the earthworm extract preparations (28-32). In the current study, we evaluated the potential hepatoprotective properties of the glycoprotein extract obtained from *E. foetidae* in two experimental models of acute and chronic hepatotoxicity.

Many different bioactive compounds such as glycoproteins, polysaccharides, peptides, amino acids, glutathione, and fibrinolytic enzymes have been extracted from earthworm (28-32). Although more studies are needed to precisely reveal the active component(s) of *E. foetidae* responsible for its cytoprotective effects, several mechanisms might be involved in its hepatoprotective properties (Figure 7).

The antioxidant capacity of the earthworms preparations has been documented in previous studies (30). It was proposed that the antioxidant capacity of the earthworm extract might be attributed to the presence of compounds such as polyphenol chemicals, glutathione, and amino acids (30) (Figure 7). Phenolic compounds are extracted from different earthworm species (30). Polyphenols are well-known examples of antioxidant agents, which act as free radical scavenging

molecules and counteract oxidative stress in biological environments (65). As mentioned, a high level of glutathione has been also detected in the coelomic fluids extracted from different worm types (30). In the current study, we found that *E. foetidae* extract supplementation mitigated liver tissue markers of oxidative stress in both acute and chronic animal models of hepatotoxicity. Hence, a significant part of the protective properties of this extract might be mediated through its antioxidant capacity (32, 66, 67).

The presence of growth factors and mitogenic compounds is another exciting feature associated with earthworms extracts (29, 68, 69). These compounds might be able to stimulate cell growth and tissue regeneration process (Figure 7). Therefore, a part of the hepatoprotection provided by *E. foetidae* extract, especially in a chronic animal model of liver injury, could be associated with the potential role of growth factors to stimulate liver regeneration (Figure 7). The effects of *E. foetidae* in regenerative medicine could be an interesting research area.

The anti-inflammatory properties of *E. foetidae* extract were also mentioned in previous experimental models (70). Inflammatory cells infiltration and the release of cytotoxic cytokines are also involved in the pathogenesis of liver injury in different experimental models (71). Hence, the anti-inflammatory properties of earthworm extract might contribute to its protective properties in acute and chronic models of liver injury.

Collectively, our data indicate that the glycoprotein extract from *E. foetidae* provided hepatoprotective properties in two experimental models of liver injury. Although more studies are needed to precisely reveal the molecular mechanisms involved in the hepatoprotective properties of this preparation, the antioxidant capacity of the extract seems to play a fundamental role in its mechanism of hepatoprotection. On the other hand, several other effects have been documented for the earthworm preparations in the literature. Hence, earthworms might be used as a cheap and convenient source of biologically active chemicals with broad pharmacological activities (Figure 7).

Acknowledgments

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Conflict of Interest

None declared.

5. References

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