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Protective Effect of Earthworms (*Pheretima hawayana Rosa and Allolobophora caliginosa Savigny)* Methanolic Extract on Acrylamide Induced Hepatorenal Toxicity

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ABSTRACT

The present study was carried out to evaluate the protective potentials of earthworm extract (EE) against acrylamide-induced hepatorenal toxicity. Forty rats were randomly divided into 4 groups (n=10). G I (control): were received distilled water (D.W); G II: rats were administered acrylamide (ACR; 5 mg/kg B.W in D.W) orally for 3 weeks; G III: rats were given EE (300 mg/kg B.W in D.W) orally for 3 weeks; G IV: rats were pretreated with EE for 3 weeks, then co-treated by EE and ACR for an additional 3 weeks. The results showed that acrylamide increased serum ALT, AST, total bilirubin, urea, and creatinine, while decreased albumin and total proteins. Acrylamide resulted in congestion of central veins and hepatic sinusoids, and hydropic degeneration of most hepatocytes. Also, ACR caused inflammatory cells infiltration in hepatic sinusoids and areas of coagulative necrosis infiltrated by inflammatory cells. Furthermore, ACR lead to alteration in kidney tissue via shrinkage of glomerular tuft with increased Bowman's space. Also, ACR lead to congestion and inflammatory cells infiltration of interstitial tissue, renal tubules with desquamated renal epithelium in their lumen forming epithelial casts. Acrylamide caused degenerative changes of some epithelial cells lining the renal tubules, other cells showed necrosis. EE recovered hepatic and renal histopathological and biochemical alterations induced by ACR. In Conclusion: Earthworm extract ameliorated acrylamide-induced hepatorenal toxicity.

Keywords: *Acrylamide, Earthworm, Rats, hepatorenal Toxicity.*

INTRODUCTION

Acrylamide (ACR) is an odorless, colorless crystalline monomer with high chemical activity. It is transported by passive diffusion throughout the body due to its hydrophilic capacity (Friedman, 2003 and Abdel-Daim *et al.*, 2014). ACR is vital manufacturing chemical used in the production of polymers and copolymers. It is created in the diet at >120 ºC procedures such as roasting, frying, cooking, and baking of foods with high carbohydrate ratio which is considered a major route of acrylamide toxicity to humans (Stadler *et al.*, 2002; Zamani *et al.*, 2017).

ACR arises via Maillard reaction between asparagine and reducing sugars when foods rich in carbohydrates are prepared at high temperatures (Alturfan *et al.*, 2012; Hamdy *et al.*, 2012; Zhang *et al.*, 2012). The exposure to acrylamide occurs via different routes, including inhalation of ACR particles through airways and

direct contact with the toxic substance (Fennell *et al*., 2004). Nowadays, ACR is formed in preserved diets, which should give attention to its possible toxicity (EL-Bohi *et al*., 2011). ACR has a neurotoxic, genotoxic, and carcinogenic effects (Dearfield *et al*., 1995; Favor and Shelby, 2005). ACR-induces toxicity via oxidative stress and oxidative damage of DNA, proteins, and cell membranes (Mannaa *et al*., 2006; Mansour *et al*., 2008).

ACR causes oxidative stress in plasma, liver, testis, brain and kidney of animals (Yousef and El-Demerdash, 2006; Prasad, 2014). ACR induces hepatocyte oxidative damage (Newairy *et al.,* 2007; Kovac *et al.,* 2015; Li *et al.,* 2018) and alters CYP2E1 expression in the liver (El-Bohi *et al.*, 2011). It was found that ACR has a high binding capacity to kidney, so ACR and its metabolites accumulate in kidneys (Miller *et al.,* 1982; Sumner *et al.,* 1997). The sub-acute levels of acrylamide cause a significant pathological change in the kidney such as acute tubular necrosis with no change in body weight or LDH activity (Rajeh and Al-Dhaheri, 2017).

Lots of people in various traditional organizations have pointed to the use of medicinal plants to treat their diseases due to there is a global shift towards the usage of a natural product than artificial products (Emerit *et al.,* 2012). In these regards, substances with antioxidant properties have recently been given extraordinary attention as probable therapeutic and preventative agents (Emerit *et al.,* 2012). Also, Natural herbal products protect against toxic side effects and are used as chemotherapeutic agents due to their antioxidant activity (Ajith *et al.,* 2007; Sak, 2012). Earthworm is considered as herbal medicine (Samatra *et al.,* 2017) from animal origin (Balamurugan *et al.,* 2009).

Earthworm, s origin is soil and has a dense nutritional content (Ismail *et al.,* 1992; Cooper, 2005). Earthworm plays an important role in nourishing soil fertility (Balamurugan *et al.,* 2008) and affect plant communities as they develop conditions for plant growth by producing persistent soil structures enriched by nutrients (Mudrák and Frouz, 2018). Earthworm is used for feeding livestock and cultured fish, also have been used widely as a fishing bait (Ganesh *et al.,* 2003; Taboga, 1980).

Many people all over the world used earthworms for thousands of years for their therapeutic benefits by extracting and using their biologically active compounds (Ranganathan, 2006) including anti-inflammatory, antitumor, antibacterial and anti-oxidative effects (Balamurugan *et al.*, 2007; Li *et al.,* 2011; Aldarraji *et al.,* 2013). Previous studies have shown that earthworms exhibit antipyretic, antispasmodic, detoxic, diuretic, antihypertensive, anti-allergic, anti-asthmatic, antimicrobial, anti-ulcer activities and have beneficial pharmacological activities such as peripheral nerve regeneration (Chen *et al.,* 2010), bone regeneration (Fu *et al.,* 2014), wound healing (Deng *al.,* 2018), anti-coagulative and fibrinolytic activity (Mihara *et al.,* 1991; Hattab *et al.,* 2015), hepatoprotective (Balamurugan, 2007), antiapoptosis (Han *et al.,* 2014; Jones *et al.,* 2016), antimicrobial, and anticancer effects (Cooper *et al.,* 2004). Therefore, the current study aimed to evaluate the protective potential of earthworm extract on hepatorenal toxicity induced by acrylamide.

MATERIAL AND METHODS

Chemicals:

Acrylamide (ACR) (L03670) and methanol (L21270) were obtained from El-Gomhouria company, Cairo, Egypt (23 Al-Swah St. Al-Ameria, Cairo). ACR is in the form of crystalline solid at room temperature, with molecular formula is CH2=CH–CO–NH2.

Preparation of Earthworm Extracts (EE):

The worms were purchased from El-Bagour, El-Menoufia. The worms (2.83 kilograms) were washed using running tap water to remove the mud particles from their surfaces. Earthworms were soaked at room temperature in distilled water for 6hrs with periodically changing D. W every 2hrs until their digestive systems became clean **(Samatra *et al.,* 2017).** Then earthworms were taken and dried with clean tissue paper, killed via cutting with scissors (Omar *et al.,* 2012) and left to dry up under shade. After dryness, earth worms were milled to obtain a coarse powder (582.7 g). The obtained powder was soaked in 80% methanol solution and kept at room temperature in a dark place for 72hr with periodical shaking. Then, the contents were filtered using filter paper (11cm, CAT. NO

1102090) and the filtrate was collected and evaporated until a soft mass was obtained. The extracts were thoroughly air dried to remove all traces of the solvent. The obtained extract was weighed (33.44 g).

Animals:

A total number of 40 male albino rats of body weights ranged from 120 to 150 g were purchased from Al-Zyade experimental animal production center, Giza, Egypt and were used for this study. The animals were housed in polypropylene cages at the animal facility of the Faculty of Veterinary Medicine, University of Sadat City, Egypt at 22^{o}c±2 temp. with good ventilation, 12h light/12h dark cycle. They were provided with balanced ration and clean water *ad libitum*. Before the onset of the experiment, animals were kept under observation one-week acclimatization. All procedures were approved by the Animal Care Committee of University of Sadat City.

The diet:

The rats offered a commercial balanced ration pellet (At Al wady Company, Egypt). Pellets ingredient were performed according to **A. O. A. C. (1980) and National research council (1989).**

Experimental design:

Forty rats were randomly divided into 4 experimental groups of 10 rats each and designated as follows:

G I: Rats were provided with a commercial diet and water and received orally 0.5 mL distilled water (vehicle of earthworm extract and for ACR) for 5 days per week for 6 weeks and served as control group (**C**).

G II: Rats were administered acrylamide (ACR) at a dose rate of 5 mg/kg B.W according to (Wang *et al.,* 2010) (dissolved in D.W) orally by gastric gavage for 3 weeks starting from 4th week to 6th week of the experiment for 5 days per week**.**

G III: Rats were given earthworm extract (EE) at a dose rate of 300 mg/kg B.W according to (Balamurugan, 2007) (dissolved in D.W) orally for 3 weeks starting from the 4th week to 6th week of the experiment for 5 days per week.

G IV: Rats were pretreated with earthworm extract as in group III for 3 weeks starting from 1st to 3rd week of the experiment; then they were co-treated with earthworm extract together with acrylamide as in group II for additional 3 weeks starting from the 4th week to the 6th week of the experiment.

Sampling:

Collection of blood samples:

At the end of the experimental period and after 12hr- fasting, animals were anesthetized with Diethyl ether (DEE). Blood samples were collected from the inner census of the eye using heparinized capillary tubes. Blood samples were collected in a glass tubes and left for clotting at room temperature, followed by centrifugation at 3000 rpm for 15 minutes. The clear supernatant serum was stored at -20^{0}C until used for investigation of biochemical/hormonal parameters.

Tissue samples:

Rats were scarified at the end of the experiment by cervical dislocation. Liver, and kidneys were picked up, washed by saline. Liver and kidney tissues were kept in 10% neutral formalin and used for histopathological examination.

Biochemical analysis:

Biochemical analysis of serum ALT, AST, total protein, albumin, bilirubin, urea, and creatinine using kits from BioMed Diagnostics (Giza, Egypt).

Histopathological examination:

Following necropsy Liver, and Kidney were rapidly fixed in neutral buffered formalin solution (10%). The fixed specimens were trimmed, washed, dehydrated in ascending grades of ethanol, cleared in methyl benzoate and embedded in paraffin. 4-µm-thick sections were cut from paraffin blocks using microtome (LEICA RM 2135). The sections were then deparaffinized with xylene and rehydrated with alcohol and water then, routinely stained by hematoxylin and eosin (H & E) stain (Sigma-Aldrich) according to Bancroft and Gamble (2008). Stained slides were examined under light microscopy using a digital Leica photomicroscope (LEICA DMLB, Germany).

Statistical analysis:

All data were presented as means ± standard errors (SE). Statistical significances were determined by one-way ANOVA according to **Snedecor and Cochran (1967).** All statistical analysis was performed using SPSS (Statistical Package for Social Sciences) Version 16 released in 2007 with $P< 0.05$ regarded as statistically significant.

RESULT:

Earthworm extracts (EE) ameliorated changes on serum biochemical marker induced by acrylamide (ACR):

Biochemical analysis of the serum revealed that administration of ACR (G II) significantly increased ALT, AST, total bilirubin, urea, and creatinine while significantly decreased total protein and albumin compared to the control group (GI) (Table 1). Rats pretreated with EE then co-treated with EE and ACR in (G IV), showed a significantly reduced ($p<0.05$) serum activity of ALT, AST, total bilirubin, urea, and creatinine while significantly increased serum levels of albumin and total protein compared to the ACR group (GII). Administration of EE alone in the (G III) had no significant effects on the serum activity of ALT, AST, and serum level of Total bilirubin, Albumin, Total protein, Urea, and Creatinine compared to the control group (GI).

Earthworm extracts (EE) modified the ACR induced Histopathological alterations in rat liver:

Fig.1 illustrates the histopathological abrasions of rat liver in all treated groups. Rats in control group (GI) showing normal histologic architecture of central vein and hepatic cords (fig. 1A). While Acrylamide group (GII) showing congestion of central vein and hepatic sinusoids, hydropic degeneration of most hepatocytes, inflammatory cells infiltration in hepatic sinusoids and area of coagulative necrosis infiltrated by inflammatory cells (fig.1B). Earthworm extract group (GIII) showing normal histologic architecture of liver tissue as control group (fig.1C). Rats co-treated with EE+ACR (GIV) showing normal hepatic cords, congestion in central vein and hepatic sinusoids and activation of Kupffer cells (Kupffer cells hyperplasia) (fig.1D).

Earthworm extracts (EE) ameliorated ACR induced Histopathological changes in rat kidney:

Fig.2 demonstrates the histopathological abrasions of rat kidney in all treated groups. Rats in Control group (GI) showing normal histologic architecture of glomeruli and renal tubules of kidney (fig.2A). Whereas, Acrylamide group (GII) showing shrinkage of glomerular tuft with increased Bowman's space, congestion & inflammatory cells infiltration of interstitial tissue, renal tubules with desquamated renal epithelium in their lumen forming epithelial casts, degenerative changes of some epithelial cells lining the renal tubules, other cells showed necrosis (fig.2B). Earthworm extract group (GIII) showing normal histologic architecture of renal tissue as control group (fig.2C). Rats co-treated with EE+ACR (GIV) showing normal glomeruli and nearly normal renal tubules except some tubules showed desquamation of some renal epithelium in their lumen (fig. 2D).

Table (1): Effect of Acrylamide and /or Earthworm extract on serum liver and kidney functions biomarkers.

Parameter	GI	GII	GIII	GIV
ALT (U/mL)	44.78±0.72 ^{b}	53.50±0.61 ^{a}	46.71±0.21 ^{b}	45.23±0.69 ^{b}
AST (U/mL)	71.19±1.66 ^{b}	88.83±1.98 ^{a}	71.67±0.87 ^{b}	75.84±0.60 ^{b}
Albumin (g/dL)	3.38±0.07 ^{a}	2.78±0.03 ^{b}	3.31±0.04 ^{a}	3.30±0.02 ^{a}
Total protein (g/dL)	6.454±0.12^{a}	5.3438±0.26^{b}	6.173±0.08^{a,c}	6.0963±0.09^{c}
Total bilirubin	0.32±0.01 ^{b}	0.56±0.01 ^{a}	0.36±0.01 ^{b}	0.28±0.01 ^{c}
Creatinine (mg/dL)	1.00±0.02 ^{b}	1.33±0.03 ^{a}	1.01±0.03 ^{b}	1.08±0.03 ^{b}

Urea (mg/dL)	37.59±0.58 [b]	45.51±2.06 [a]	37.93±0.40 [b]	38.31±0.31 [b]

Mean value ± SEM (standard error of mean). The mean difference is significant at P < 0.05. The values carrying different letters in the same row were statistically different

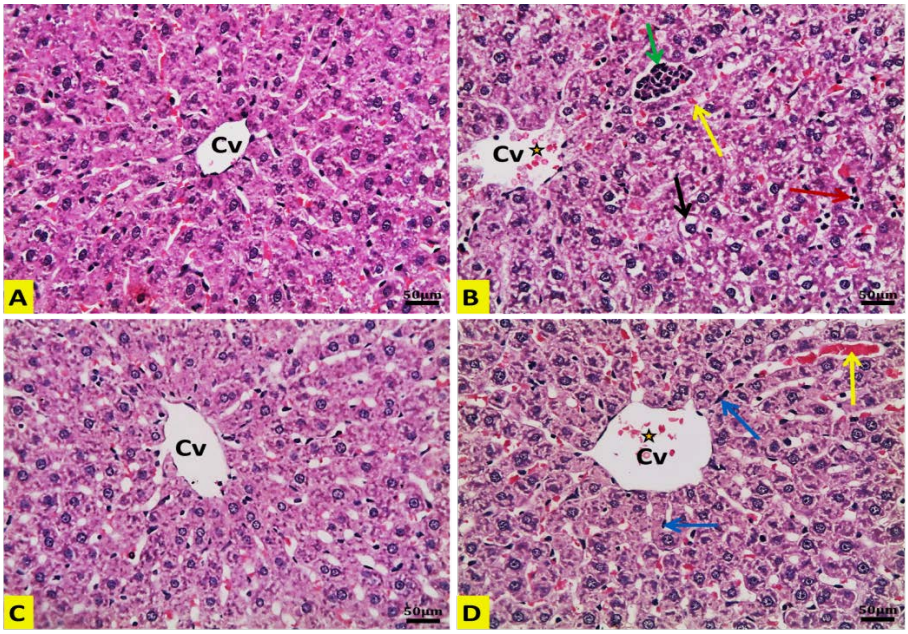

Figure 1: Histopathological photomicrographs of rat liver (H&E X_{20}): A. Liver tissues of group I; B. Liver tissues of group II; C. Liver tissues of group III; D. Liver tissues of group IV.

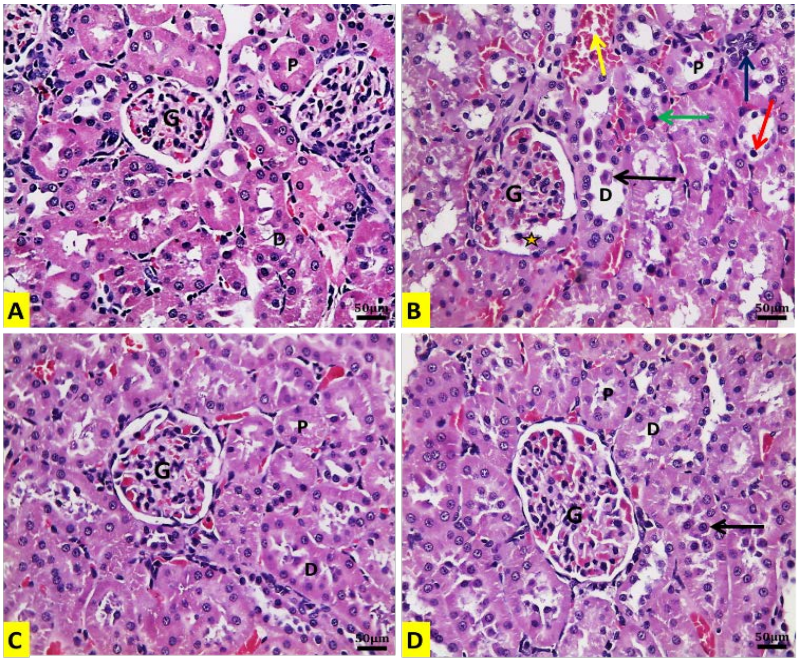

Figure 2: histopathological photomicrographs of rat kidney (H&E X_{20}): A. Kidney tissues of group I; B. Kidney tissues of group II; C. Kidney tissues of group III; D. Kidney tissues of group IV.

DISCUSSION:

Acrylamide is considered as one of the main environmental health problems (Riboldi *et al.*, 2014). Earthworm extract (EE) is broadly used in traditional medicine in China (Cooper, 2005), and practiced by a lot of peoples throughout the world (Ranganathan, 2006). Recently it is reported that earthworm paste has anti-oxidative and anti-ulcer properties (Balamurugan *et al.*, 2007 and Prakash *et al.*, 2007).

AST and ALT are cytoplasmic enzymes and act as a biomarker enzyme for liver disease. ALT, catalyses the conversion of alanine to pyruvate and glutamate and it is a good indicator of liver injury than AST (Willianson *et al.*, 1996). Bilirubin is a product resulting from the breakdown of hemoglobin (Sanjiv, 2002). The

present study revealed a significant elevation in serum activities of ALT, and AST which indicates hepatic cellular damage (Kim and Mahan, 2001). This explains the elevated serum total bilirubin, and reduced serum level of albumin and total protein. Previous studies reporting significantly increases the activities of ALT and AST in rats (Hamdy *et al.,* 2017). There was a significant reduction in serum levels of albumin and total protein in GII (ACR group) compared to control group. This could be attributed to the disturbances in protein metabolism and indicates hepatic cell dysfunction and agree with the results of Mahmood *et al.* (2015) and Belhadj Benziane *et al.* (2019). These results disagree with shrivastava *et al.* (2017), and (2018) who reported a significant increase in albumin level due to loss of the integrity of liver cells and hepatocellular dysfunction caused by ACR. Our results confirm previous studies which showed an increase in total bilirubin indicating liver dysfunction (Uthra *et al.,* 2017). The present study revealed that EE significantly reduced serum level of ALT, and AST, and the level of total bilirubin while significantly increased serum levels of total protein and albumin. This indicates that EE was able to ameliorate the toxic effect of ACR and proves the hepatoprotective effect of EE. The result is harmonized with other finding showing that administration of EE had hepatoprotective effect against injury caused by paracetamol and evidenced by decreases in serum ALP, AST, and ALT activities, bilirubin and liver levels of thiobarbituric acid reactive substances (TBARS) (Balamurugan *et al.,* 2008). Moreover, our findings agree with those of Sadek *et al.* (2016) who reported that EE restores the hepatic tissue injury caused by silicon dioxide nanoparticles (SiNPs) and decreases serum activities of AST, ALT, GGT, and ALP, and levels of total and direct bilirubin. Parallel to biochemical changes acrylamide lead to varying degree of damaging of hepatic cell. In this study, ACR cause congestion of central vein and hepatic sinusoids, hydropic degeneration of most hepatocytes, inflammatory cells infiltration in hepatic sinusoids and area of coagulative necrosis infiltrated by inflammatory cells. These results matched with results of Rawi *et al.* (2012) who reported that ACR caused degeneration and even apoptosis in some hepatocytes. Also, the results are in line with those of Hamdy *et al.* (2017) who reported that ACR lead to degenerative changes in numerous hepatocytes, many pyknotic nuclei, and prominent mononuclear cell (lymphocyte) infiltrates were also noted in between hepatocytes and around the central vein. This alteration greatly improved as a response to EE treatment and these results agree with the finding of Sadek *et al.* (2016) that reported that the analysis of EE revealed high levels of essential and nonessential amino acids and the precursor amino acids of GSH which are glutamine, glycine, and cysteine. This important due to it was reported by Lieber *et al.* (1990) and Anuradha and Vijayalakshmi (1995) that the administration of those amino acids such as cysteine and methionine increase the levels of antioxidants and minimizes the oxidative stress.

Kidneys are responsible for excreting metabolic waste products and preserve the body homeostasis (Edwards, 2010). Detection of serum urea and creatinine levels are a screening tests for renal function. Urea and creatinine are nitrogenous end products of metabolism, urea is metabolite derived from tissue protein turnover and from dietary protein, but creatinine results from creatine catabolism in muscle (Walker *et al.,* 1990). In the present study, there was a significant increase in the levels of serum urea and creatinine in the ACR-treated group which indicates an impaired renal function. These studies are in agreement with the findings reporting that there is a significant increase in serum urea and creatinine levels in rats treated with ACR indicating renal dysfunction (Khalifa *et al.,* 2016; Hamdy *et al.,* 2017; Shrivastava *et al.,* 2017 and Acaroz *et al.,* 2018). Also, another study reported an elevation in serum urea and creatinine level in response to ACR treatment and attributed this to ACR toxicity causing damage of the brush border epithelial cells of the kidney and become impermeable to urea and creatinine thereby leading to their elevation in blood (Uthra *et al.,* 2017). In group pretreated with EE and then co-treated with EE and ACR, there was a decrease in serum urea and creatinine levels. These results indicated that EE was capable of ameliorating the impaired kidney functions caused by ACR. These results are in agreement with previous studies reported that

EE administration (300 and 500 mg/kg, i.p) reduced serum creatinine, BUN and lipid peroxidation in kidney tissue significantly (Jamshidzadeh *et al.,* 2016).The protective properties of this extract are mostly to be mediated by its antioxidant capacity. Previous studies of Grdisa *et al.* (2001) and Balamurugan *et al.* (2008) revealed that earthworm's glycoprotein extract showed an antioxidant activity. Matching with biochemical results the histopathological examination of kidney tissues of ACR-treated rats showed renal tissues damage as, shrinkage of glomerular tuft with increased Bowman's space, congestion & inflammatory cells infiltration of interstitial tissue, renal tubules with desquamated renal epithelium in their lumen forming epithelial casts, degenerative changes of some epithelial cells lining the renal tubules, other cells showed necrosis. This results in line with the findings of Rajeh and Al-Dhaheri, (2017) and Hamdy *et al.*, (2017). These damages are improved on treatment with earthworm extract due to antioxidant properties of EE (Jamshidzadeh *et al.,* 2016) and proved that EE has renoprotective effect against ACR toxicity. It was reported that EE have growth factors and some mitogenic compounds (Grdisa *et al.,* 2004) which motivate cell replication and regeneration. This might explain the renoprotective properties of EE.

CONCLUSION:

In conclusion earthworm methanolic extract has a protective effect against acrylamide induced hepatorenal toxicity in rats.

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