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Toxicity evaluation of earthworm powder (*Eudrillus euginae*) in wistar male rats

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ABSTRACT

Objective: To evaluate the acute toxicity effects of earthworm powder (EWP) obtained from Eudrillus euginae on wistar male rats. **Methods:** The animals are treated orally with EWP at the doses of 100 mg/kg, 200 mg/kg and 300 mg/kg bodyweight daily for 12 days. **Results:** There was no significant mortality and changes in body weight noticed at all the doses tested. No gross significant changes observed in the hematological indices (HB, RBC and WBC), Hepatic, serum markers (AST, ALT, ALP, and protein) and renal indices (Urea, Uric acid and Creatinine). The EWP did not show any significant effects in the clinical signs, behavioral changes and examined toxicological endpoints in experimental animals. The histopathological studies provide supports to the safety data of above parameter of EWP dosing. **Conclusions:** Thus, it can be inferred from the present study that EWP (Eudrillus euginae) is devoid of toxic effects in rats and suggested to utilize the EWP as novel medicine.

1. Introduction

Although Wallace (1853, 1889) reported about the inclusion of earthworms in diets of Amerindian populations little is known about the nutritive value of these edible invertebrates[1]. Earthworms are found virtually worldwide and live in almost any type of soil that contains the right amounts of moisture and organic particles. Earthworms are of various sizes and colors[2]. The earthworm is a widely used Chinese herbal medicine[3]. It has dense nutritional content because of their soil based origin[4]. Extracting medicinal compounds from the earthworm has traditionally been practiced by indigenous people throughout the world, more particularly in Asia[5]. Previous earthworm studies have shown its antimicrobial[3], hepatoprotective[4], anticancer and scar wound healing characteristics[6,7]. The anti-inflammatory activity together with antioxidant properties seems to be due to the high polyphenolic content in earthworm tissue[8]. Moreover, crude earthworm extract has a thrombolytic effect that could significantly promote blood circulation to remove stasis[9]. Lumbrokinase extracted from the earthworm has been used to treat stroke and cardiovascular diseases[10]. However, it is only during the past few decades, with the development of biochemical technologies, that research on the pharmaceutical effects of earthworms has been initiated[11].

In order to support an application for a clinical trial or for the registration of a new drug, it is necessary to satisfy legislation that requires that certain data should be produced from a variety of toxicological investigations that show the safety profile of the compound to which humans may be exposed. Therefore, in the majority of cases of evaluation of the toxicity of most substances, rodents and non-human primates are first used in preclinical animal safety studies before further studies are done in humans. These animals are mainly used because of their biological similarity to humans that allows them to be regarded as the suitable metabolic models for humans in a broad range of investigations[12].

Though pharmacological role of different natural herbal products and formulations has been reported, similar studies have not been made on tissues of animal origin, especially earthworms. Since studies on the medicinal value of indigenous earthworms are limited the present study was aimed to evaluate the acute toxicities of earthworm powder (EWP) obtained from Eudrillus euginae on male wistar rats.

2. Materials and methods

2.1 Collection of earthworm

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The earthworm namely Eudrillus euginae was collected from Aarthi farms, Kondegoundampalayam Village, Pollachi Taluk, Coimbatore District, Tamil Nadu, India. The species were cultured in Kongunadu Arts and Science College Premises, Coimbatore - 641 029, Tamil Nadu, India, for further use. The earthworms were harvested whenever needed.

2.2 Extraction and preparation of the sample

The earthworms were washed with running tap water to remove any dirt from body surface. The earthworm was kept in 0.65% NaCl at room temperature for 1-2 h with few changes of solution until their digestive systems were clean. Animals were taken out of the solution and minced with scissors. Three grams of earthworm tissue were homogenized in 40 mL of chloroform-methanol (v/v) solution and left overnight at 4℃. The following day, 16 mL of distilled water was added to the homogenate. It was mixed and centrifuged at 2460 r/min for 10 min. Three clearly visible layers were obtained. The upper, water/methanol layer was taken out by pipette and evaporated on a rotary evaporator until methanol was left. An opalescent fluid, pH 7, was obtained. It was freeze dried earthworm powder (EWP) was kept at 4℃ until use[13].

2.3 Experimental animals

Healthy Wistar albino male rats of 6 weeks of age weighing approximately 100-110 g were procured from Small Animal Breeding Centre, Kerala Agricultural University, Mannuthy, Thrissur. The institutional animal ethic committee (IAEC) approved the research. The rats were grouped and housed in polypropylene cages and maintained in standard laboratory conditions (temperature 25±2℃) with dark / light cycle (14 / 10 h). They were acclimatized to laboratory conditions for 10 days prior to the commencement of the experiment. They were allowed for free access to standard dry pellet diet and water.

2.4 Experimental design

The acute toxicity study was aimed to establish the therapeutic index of pharmacologically effective dosage and performed the primary screening. The study was carried out to determine the effective dose of Earthworm Powder (EWP) without any side effects. After the adaptation period, the animals were divided into four groups of six animals in each group.

Group I: Normal control rats

Group II: Rats fed orally with EWP (100 mg / kg body weight for 12 days)

Group III: Rats fed orally with EWP (200 mg / kg body weight for 12 days)

Group IV: Rats fed orally with EWP (300 mg / kg body weight for 12 days)

2.5 Mortality and clinical signs

During the 12 days of dosing period, all the animals were observed daily for clinical signs and mortality patterns once before dosing, immediately after dosing and up to 4 h after dosing.

2.6 Body weight analysis

The body weight of each rat was assessed using a sensitive balance during the acclimatization period, once before commencement of dosing, once weekly during the dosing period and once on the day of sacrifice.

2.7 Haematology, biochemical and renal indices

After the experimental regimen, the animals were sacrificed by cervical decapitation under mild chloroform anesthesia. Blood was collected into clean tubes by incision made in the jugular vein. The free flow blood was assayed for hemoglobin[14], red blood cells (RBC) and white blood cells (WBC) in the control as well as treated groups[15]. Serum was prepared from the collected blood. The 10% liver homogenate and serum were used for the estimation of various biochemical parameters such as protein[16], aspartate aminotransferase (AST), alanine amino transferase (ALT)[17], and alkaline phosphatase (ALP)[18] were quantified. Superoxide dismutase (SOD)[19], catalase (CAT) [20], lipid peroxidation (LPO)[21]. The renal indices like urea, uricacid and creatinine were assayed in serum alone[22-24]. A section of the liver from each group was analyzed for histopathological examination.

2.8 Source of chemicals

The chemicals used in the present study were of analytical reagent grade. It was purchased from SD fine chem.., HiMedia and Qualigens, India.

2.9 Statistical analysis

The data were expressed as mean± SD for six animals in each group. Total variation present in a set of data were estimated by one way analysis of variance (ANOVA) followed by the analysis of levels of significance between different groups based on ANOVA. The difference among means was analyzed by DMRT at 5% level ($P< 0.05$).

3. Results

Acute toxicity has been defined as the ability of a substance to cause severe biological harm or death soon after a single exposure or dose; or any poisonous effect resulting from a single short-term exposure to a toxic substance[25].

The sighting study did not result with any clinical signs of toxic effect at all, the dose (100, 200 and 300 mg/kg bodyweight) level tested. Clinical signs throughout the study indicated that none of those showed signs of toxic effect such as changes on skin, eyes color, behavior pattern, urine and diarrhea. The toxic study did not result in any mortality of EWP dosing and no toxic effect was observed throughout the dosing period when compared to control rats.

The experimental rats gained weight throughout the duration of the study. From Table 1 it is evident that there is a progressive increase in body weight at doses of 100, 200 and 300 mg/kg of male rats during 12 days of administration of EWP. The body weight of the treated rats were not significant different as compared to the control rats.

Table 1.
Average Body weight of Wistar rats

Treatment	Initial	On the day of sacrifice	Mean body weight (g)
Group I	110	160	50±1.5
Group II	110	165	55±1.0
Group III	100	160	60±1.0
Group IV	110	165	55±1.0

Values are expressed as mean ± SD (n = 6).

Table 2.
Effects of EWP Haematological profile of wistar rats.

Treatment	HB*	RBC**	WBC***
Group I	13.65 ± 0.015c	3.32 ± 0.005d	5.26 ± 0.008d
Group II	13.61 ± 0.010d	3.70 ± 0.062a	5.84 ± 0.008 cd
Group III	13.75 ± 0.008ab	3.54 ± 0.006c	6.74 ± 0.013ab
Group IV	13.73 ± 0.036ab	3.65 ± 0.009b	5.92 ± 0.018ab

Values are expressed as mean ± SD (n = 6).* $P< 0.05$
Groups compared: Group I vs Group II, Group III, Group IV
Units: * – g/dL, **–Number of RBCs/mm3, ***– Number of WBCs/ mm^3.

Table 3.
Effect of EWP on enzymatic and nonenzymatic antioxidants

Treatment	SOD*	CAT**	LPO***
Group I	4.62 ± 0.008a	8.67 ± 0.01a	5.73 ± 0.030a
Group II	3.86 ± 0.013b	7.33 ± 0.01d	4.98 ± 0.008b
Group III	4.52 ± 0.017c	8.03 ± 0.01 b	4.87 ± 0.023 c
Group IV	3.98 ± 0.008d	7.95 ± 0.01 c	4.95 ± 0.044d

Values are expressed as mean ± standard deviation, (n = 6).*($P <$ 0.05) significant value.
Groups compared: Group I vs Group II, Group III, Group IV
Units: * – inhibition of 50% nitrite formation/min/mg protein
** – μ mol of H_2O_2 decomposed/min/mg protein
*** – nmol/mL

Table 4.
Renal profile of rats treated with EWP

Treatment	Urea*	Uric acid*	Creatinine*
Group I	25.36 ± 0.01c	1.77 ± 0.01b	0.59 ± 0.005ab
Group II	25.65 ± 0.01b	1.54 ± 0.01d	0.57 ± 0.013 bcd
Group III	25.74 ± 0.02a	1.66 ± 0.01c	0.56 ± 0.022 bcd
Group IV	24.88 ± 0.01d	1.86 ± 0.02a	0.58 ± 0.008 abc

Values are expressed as mean ± standard deviation, (n = 6).*($P <$ 0.05) significant value.
Groups compared: Group I vs Group II, Group III, Group IV
Units: * – mg/dL

The effect of EWP on haematological parameters like Hb, RBC and WBC were assessed after dosing and represented in Table 2. No significant variation for Hb, and RBC were observed with the tested doses. The white blood cell was found to be non significantly increased ($P<0.05$) at the doses of 200mg/kg and decreased in 300mg/kg. With the exception of a transient change in WBC count there were no significant alterations in the hematological parameters.

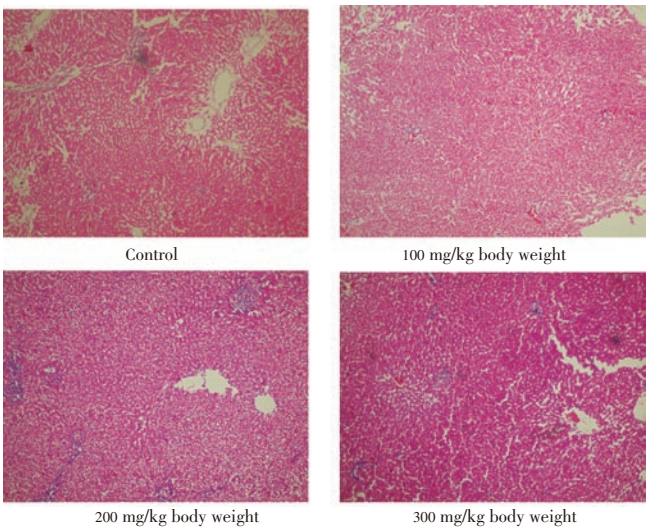

Figure 1. Histopathological artitecture of liver in control and experimental rats.

The activities of antioxidants like SOD, CAT and LOP were estimated in liver of control and experimental rats were depicted in Table 3 From the table it is evident that the antioxidant profile was found to be non significant decrease in the tested doses (100 mg/kg and 300 mg/kg bodyweight) when compared to control. In contrary increased antioxidant status was noticed in group III animals (200 mg/kg bodyweight). The LPO was remained same as control in all groups.

The serum levels of urea, creatinine and uric acid at dose levels of 100, 200 and 300mg/kg shows non-significant ($P<0.05$) increase compared with control were given in Table 4 respectively. The levels of urea were found to maximum with all the doses tested compared to uric acid and creatinine.

Table 5 represents the toxic indices such as AST, ALT, ALP and protein which was performed in both serum and liver of control and experimental rats. A non-significant increase in the levels of AST, ALT, and ALP in serum and liver were observed in group II and group III (100 and 200mg/kg bodyweight) as compared with the control. But the significant elevation of ALT was found to be present in 300 mg/kg. There were no significant changes noticed in the protein levels in all the doses tested in both serum and liver.

A section of the liver of all the rats in each group were

Table 5 .
Activity of hepatic function indices in serum and liver of control and experimental groups of rats

Groups	Serum				Liver			
	AST*	ALT*	ALP*	Protein**	AST*	ALT*	ALP*	Protein**
Group I	10.93 ± 0.01ab	8.73± 0.01cd	4.20 ± 0.15abc	0.20 ± 0.00ab	2.56 ± 0.02ab	2.96 ± 0.01bc	2.73± 0.01bc	0.56 ± 0.00d
Group II	10.97 ± 0.01ab	8.84± 0.01cd	4.43± 0.28abc	0.20 ± 0.00abc	2.53± 0.01cd	2.95± 0.01bcd	2.60± 0.11b	0.57 ± 0.00c
Group III	11.33 ± 0.02a	8.96± 0.02bc	4.43 ± 0.22abc	0.20 ± 0.00abc	2.58± 0.02ab	2.97 ± 0.02bcd	2.71± 0.01bc	0.57 ± 0.00b
Group IV	11.13 ± 0.09b	9.36 ± 0.28a	4.94 ± 0.01d	0.20 ± 0.00abc	2.59± 0.01cd	3.12± 0.02a	2.85 ± 0.01a	0.58 ± 0.00 a

Values are expressed as mean ± SD(n = 6).*(p < 0.05) significant value.
Groups compared: Group I vs Group II, Group III, Group IV
Units *– μ moles of pyruvate liberated/ unit time/ liter, **– mg/g

removed and grossly examined for tissue toxicity (Figure 1).

4. Discussion

At the dose (100, 200 and 300 mg/kg bodyweight) levels tested, no untoward clinical signs were observed in the rats. There were no changes in the nature of stool, urine and eye colour of all the animals. No mortality was observed in the different groups of rats that received EWP orally for 12 days. The EWP treated rats were found to gain their weights through out the dosing period. The differences in body weight and body weight gain may have resulted from physiological variation in rats such as food intake, and metabolism. Furthermore, neither morbidity nor disease was observed during the entire experimentation period. Besides, the physical examination during the experimental period indicated that all animals are healthy. This was confirmed by the general but significant increases in the body weight observed in all the treated groups throughout the study duration. When animals lose appetite (anorexia), weight loss is bound to ensue due to disturbances in carbohydrate, protein or fat metabolism[26]. The general increase in body weight observed shows that the extract possibly did not induce anorexia, an effect that could have resulted in loss of body weight.

No significant variation for Hb, and RBC were observed with the tested doses. The white blood cell was found to be non significantly increased and these non-significant changes of haematological parameters are within normal ranges[27-29]. The haemoglobin and the RBC levels were not affected suggesting that haemolytic anemia and polycythemia, (that are characterized by decreases and increases in RBC count, haematocrits and hemoglobin, respectively), were not likely to be induced by EWP. Increase in WBC may indicate the impact of EWP in boosting the immune system of treated groups. However, slight changes in WBC did not show any dose responsiveness. The levels of white blood cells, (which serve as scavengers that destroy microorganisms at infection sites, remove foreign substances and debris that results from dead or injured cells[30], were also not changed suggesting that the EWP was also not toxic to the immune system and did not affect leucopoiesis.

Antioxidants constitute the foremost defense system that limit the toxicity associated with free radicals. The reason for increase lipid peroxidation in plasma of patients with free radicals may be a poor enzymatic and non-enzymatic antioxidant defence system. Free radical-scavenging enzymes such as SOD and CAT are the first line of cellular defense against oxidative injury, decomposing O_2 and H_2O_2 before interacting to form the more reactive hydroxyl radical (SOH). These enzymes protect the red cells against O_2 and H_2O_2 – mediated lipid peroxidation[31]. Since, there was no significant increase in lipid peroxidation, which shows that EWP has adversely boosted the antioxidants.

In our study, it is observed that the blood urea, uric acid and creatinine levels were significantly increased in all the dose levels when compared to control. Blood urea nitrogen is derived in the liver protein / amino acid from diet or tissue sources and is normally excreted in the urine. In renal disease, the serum urea accumulates because the rate of serum urea production exceeds the rate of clearance. Creatinine, on the other hand, is mostly derived from endogenous sources by tissue creatinine breakdown. The plasma creatinine concentrations in normal individuals are usually affected by a number of factors such as the muscle mass, high protein diet and catabolic state, thus serum urea concentration is often considered the more reliable renal function predictor than serum creatinine[32], and thus serum urea concentration is often considered the more reliable renal function predictor than serum creatinine. In collectively the EWP doses not have adverse effect on serum indices.

Many compounds are metabolized in the liver, but if too many demands are made on this organ's capacity, the continued function of its cells is no longer ensured[31]. It is known that the liver and kidneys play significant roles in various metabolic processes. The liver plays an important role in xenobiotic function and the kidneys are the main organs involved in drugs elimination, and, therefore, particularly exposed to the toxic effects of exogenous compounds[33]. It was thus important to investigate the effect of EWP on the function of these organs. The transaminases (AST and ALT) are useful enzymes as biomarkers predicting possible toxicity[34]. Any damage to the parenchymal liver cells will result in elevations in both these transaminases[35]. On the other hand, AST, ALT and ALP found in the serum and liver is of both mitochondrial and cytoplasmic origin and if it is raised that can be taken as a first sign of cell damage that lead to the outflow of the enzyme into the serum[31]. Our study was in accordance with the Pieme et al[36]; Shashi[37], who reported that the three most important and common liver enzyme in liver profile were AST, ALT and ALP which were not affected by the administration of the CP leaf extract. Furthermore, there was no effect on the levels of transaminases AST, ALT and ALP, good indicators of liver and kidney functions, respectively. Collectively, all the results suggest that the oral ingestion of the aqueous extract of EWP did not induce alterations in the serum biochemical parameters or damage to the liver of the rats.

The liver of both control and EWP treated rats (100, 200, 300mg/kg bodyweight) showed normal histological architecture. In all the groups the hepatocytes were found to be normal, with normal triads, central vein and hepatic sinusoids. Fibrosis and regenerative activity were not observed. Inflammations were not observed and blood vessels were observed to be normal. Hence, it was incurred from the present study that all the selected doses of EWP (100, 200, 300 mg/kg bodyweight) was found to be nontoxic to the experimental rats. In conclusion the results showed no macroscopic or microscopic produce acute toxicities like centrilobular degenerative changes, or necrosis at all the doses tested.

Acute toxicity studies of EWP did not show any significant effects in the clinical and behavioral profile, biochemical profile, renal indices and blood parameters of experimental animals. The EWP does not show any adverse toxic effect on the experimental animals at all the tested doses. Over all the results of this study provide valuable preliminary data on the toxicity profile of EWP that will be useful for the planning of future pre-clinical and clinical studies of this earthworm powder (EWP) of Eudrillus euginae as medicine

Conflict of interest statement

We declare that we have no conflict of interest.

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