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A STUDY ON *IN VIVO* EVALUATION OF HAEMOSTATIC POTENTIAL OF EARTHWORM POWDER (*Eudrilus eugeniae*)

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ABSTRACT

Homeostasis represents the balance between the process of coagulation and fibrinolysis. In the present study the earthworm powder (glycolipoprotein mixture) from Eudrilus eugeniae was extracted and tested for its haemostatic effect in Wistar rat model. The 100 mg/kg of the body weight dose was fixed after performing the acute toxicity studies in Swiss albino mice. The haemostatic potential of the earthworm powder was determined by studying the bleeding time and clotting time. The bleeding time was recorded to be 201 ± 2.2 seconds and clotting time was 165± 1.05 seconds at the dose of 100 mg/ kg of the body weight, thus indicating a decrease ($p< 0.05$) in clotting parameter compared to its control. Thus these findings would help to find a new pharmacological product in the haemostatic disorders in the human and veterinary field.

***KEYWORDS**: Acute toxicity study, Earthworm powder, Eudrilus eugeniae, glycolipoprotein, haemostasis.*

INTRODUCTION

Haemostasis is a complex process which changes blood from a fluid to a solid state and is a process that prevents excessive blood loss in the body. The exploration of compounds that facilitate the process of haemostasis is of medicinal importance as this is considered to be a life saving process (1). The injury to tissues results in bleeding with the subsequent activation of acute inflammatory reactions. So the bleeding from the damaged blood vessels in the injured tissue must be arrested through the process of haemostasis. The report (2) that the natural bioactive molecules are crucial in modern theraeutics, and search for them is a very attractive strategy. Earthworms are a common research systemfor investigation because they possess many biological activities such as regeneration, immunological recognition and memory (3). According to (4) G-90 is a macromolecular mixture with glycolipoprotein characteristics and is obtained from the tissue homogenate of the earthworm, *Eisenia*. *Foetida* G-90 has strong anticoagulative and fibrinolytic activities in vitro. It is already known that the phylum *Annelida* proceses substances with fibrinolytic and anticoagulative actvities. The phylogenetically close leeches and earthworms also exhibit such physiological activities.

The source of biological material in our investigation was the culture of earthworm species *Eudrilus eugeniae.* The earthworm species, *Eudrilus eugeniae,* commonly referred to as the West African night crawler, occurs all over the world but mostly in West African regions (5), (6). *E. eugeniae* is considered to be a suitable species for our study due to its rapid growth rate, larger size (> 12 cm), quick attainment of sexual maturity, shorter incubation period and can withstand the room temperature. Thus our study was designed for the in vivo evaluation of the haemostatic potential of earthworm powder (glycolipoprotein mixture) from the wet tissue of *Eudrilus eugeniae* in Wistar rats.

MATERIALS AND METHODS

Preparation of earthworm (glycolipoprotein mixture) powder

The earthworm *Eudrilus eugeniae* were collected from the vermiunit of Department of Microbiology, Nehru Arts and Science College, Coimbatore, Tamil Nadu. The earthworm powder was obtained by following the procedure (7) with little modifications. The earthworms were fed with tissue paper so as to clean their own alimentary canal and then they were kept in 0.65% sodium chloride solution for 1-2 to changes of solution until their digestive system was completely clean. Three grams of earthworm tissue was homogenized with 40ml of equal part of chloroform: methanol solution and left at 4°C overnight. The following day, 16 ml of distilled water was added to the homogenate. It was mixed and centrifuged at 2800 g for 10 minutes and three clearly visible layers were obtained. The upper, water/methanol layer was-taken out by pipette and evaporated on a rotavapour until there was no more methanol. An opalescent fluid (pH 7) was obtained. It was lyophilised and the powder was stored at 4°C for further use.

Pharmacological Tests

Animals

Adult Swiss albino mice (weighing 18-25 g) and Wistar rats (200- 250 g) of both sexes were obtained. The animals were housed in polypropylene cages, in a temperature controlled environment (23 ± 2ºC) and 50 – 60 % humidity. Lighting was controlled to supply 12 h of light and 12 h of dark for each 24-hour period. The animals were fed with standard laboratory animal food pellets with water *ad libitum*. The dosing volume did not exceed 1 ml/ 100 g of the body weight. The institutional ethical committee of KMCH College of Pharmacy, Coimbatore, Tamilnadu, India approved the protocol for these experiments under number KMCRET/PhD/05 /2012-13.

Acute Oral Toxicity Studies

The acute oral toxicity test was performed as per Organization for Economic Co-operation and Development (8) guidelines 423 on Swiss albino mice. Mice were divided into six groups of six mice (n=6) animals. They were identified by the markings using a yellow stain. One mouse was not marked and the others were marked on head, body, tail, head and body, body and tail, to ease the observation. The test substance was administered orally at a dose of 100, 500, 750, 1000 and 2000 mg/kg after fasting them for 24 hours. After the administration of test substance, food for the mice was withheld for 2 hours. Animals were observed individually after at least once during the first 30 minutes to 2 hours, periodically during the first 24 hours, and daily thereafter, for a total of 14 days. The behavioural changes of various parameters in Central Nervous System depressant, Central Nervous System stimulant and Autonomic Nervous system activity were observed.

Study of Haemostatic Parameters

Experimental design

The animals were divided into three groups (n=6): Group I was kept as control, without administration of drug, Group II received oral administration of vehicle (distilled water), Group III- received oral administration of glycolipoprotein extract powder at the dose of 100 mg/kg (least dose in acute toxicity).

Determination of bleeding time

The bleeding time was determined using a modified tail cutting method, as described previously (9), (10), (11). The tails of all animal were disinfected by cleaning with methylated spirit. The tip of the tail was quickly cut using a disposable lancet and the stopwatch was started as soon as bleeding started. The cut was dabbed with filter paper every 30 s until the paper no longer stained red with blood. Bleeding time was taken as the time for the first drop of blood to show to the time when the filter paper stopped showing bloodstain (12). Bleeding time was noted at 0 min, 60min and 120min after administration of the extract.

Determination of clotting time

The blood was collected in a capillary tube by retro-orbital puncture. A stop clock was started immediately and the time taken to form thread-like structure while breaking the capillary tube was noted in seconds (13). For antithrombotic activity reactants, the (extract solution and normal saline) was added before clot formation that is immediately after taking blood in tubes. All reactions have been maintained at 37°C in water bath. The interval between the introduction of the blood and the time of clot formation was taken as the coagulation time (12).

Statistical Analysis

The data was analyzed by using SPSS software (version 16.0, SPSS).The results were expressed as mean ± standard error of mean (SEM). The comparisons were made between the treated groups by the use of single way Analysis Of Variance (ANOVA).The values of $p < 0.05$ were considered as significant.

RESULTS

The earthworm powder (glycolipoprotein mixture) (Fig. 1) was prepared and was further subjected to acute toxicity and haemostatic effect. In the acute toxicity studies the mice (Fig 2) administered with earthworm powder (glycolipoprotein) did not show any sign of behaviour changes during initial 2 hours (Fig 3) after the oral administration of the drug even at the highest dosage of 2000 mg/kg (Table 1). No mortality was observed during 14 days after the administration of the earthworm powder. The LD_{50} value was indeterminable being in excess of 2000mg/kg body weight. So, testing the extracts at a higher dose may not be necessary and the extracts were practically non-toxic. The haemostatic effect of the earthworm powder was used for evaluation using Wistar rat model. The earthworm powder showed pronounced difference in bleeding time at the dose of 100 mg/kg (201$\pm$ 1.2) at 120 minutes ($P<0.05$) compared to the control (180 $\pm$ 1.2 seconds) and the vehicle. The clotting time obtained at 100 mg/kg dose of earthworm powder was 165$\pm$ 1.05 seconds compared to its control of 120$\pm$ 1.33 seconds and the clotting time decreased compared to the control where the drug was not administered when the reading was taken at

120 minute time (Table 2). The 120 min time taken for slight increase in bleeding and clotting time may be for the absorption of orally administered earthworm powder.

DISCUSSION

The use of bioactive components from animals and plants can be used as an alternative treatment in the field of medicine and search for such biological compounds had been increasing worldwide. According to (14), haemostasis involves the spontaneous arrest of bleeding from damaged blood vessels which is important for initiation of tissue repair processes and prevention of tissue death through haemorrhage. The description on the study of the haemostatic effect of the earthworm powder from *E eugeniae* was not reported though on another species was done. The acute toxicity studies were done as per the OECD guidelines 423 and revealed that the powder did not produce any mortality throughout the study period of 48 hours and thereafter for 14 days even when the limit dose was maintained at 2000mg/kg body weight.. The medium lethal dose (LD_{50}) of the extract was found to be higher than 2000 mg/kg body weight and hence, the powder had no adverse effect, in a single dose of administration.

The haemostatic potential of the extracts were studied wherein the clotting increased significantly in the rats administered with the earthworm powder compared to the control. The bleeding time were observed to see the effect of the test material on the process of platelet's haemostatic process and was shown by the slight increase in length of bleeding time after giving the earthworm powder. The impaired haemostasis will lead to impaired wound healing and the haemostatic phase starts as soon as vessels are damaged. Platelets are the blood cells involved in coagulation (15), (16) or it may inhibit the formation of prostaglandin by the vessel walls during injury. Since bleeding time is an index used to indicate the amount of circulating platelet in blood, it then implies that the extract greatly reduced the number of circulating platelets thereby decreasing the tendency for blood to coagulate via formation of a platelet plug (one of the first processes involved in blood coagulation that is after vasoconstriction), making it less possible to occur and consequently increasing the time it takes for bleeding to stop after its initiation.

From the results obtained, there was significant increase in clotting time, thus indicating that there was an increase in one or more of the clotting factors involved in the intrinsic pathway as this is a qualitative measurement of factors involved in the intrinsic pathway. A number of studies have been conducted by various researchers to find out the herbs and natural food sources and their supplements having antithrombotic (anticoagulant and antiplatelet) effect and there is evidence that consuming such food leads to prevention of coronary events and stroke (17). The observation on the coagulation time is used to observe the effect of the powder on the formation of secondary haemostatic plug, the haemostatic process of the coagulation phase. Similar the presence of bioactive molecules in G-90 (glycolipoprotein) from *Eosenia foetida*, which could maintain the homeostasis, was examined on venous blood from the patients suffering from primary malignant tumors of different organs (tissues) and was, reported (18) earlier. Though there is only slight increase in the bleeding and clotting time which is time dependent, since the study was performed by varying the time. So to know the correct mechanism of action the same experiment should be performed for 14 days as this study is only an initial one. Further studies should be performed to characterise its mechanism so as to develop it as a thrombolytic agent. Thus our results provide the basis for future studies which should include determining the active components from the earthworm powder of glycolipoprotein fraction, responsible for the haemostatic effect.

Table 1 :- Effect of acute oral toxicity tests in mice.

Gross Behaviour	H (Head)		B (Body)		T (Tail)		HB (Head Body)		BT (Body Tail)		NM (Not Marked)	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
CNS DEPRESSION												
Hypo activity	A	A	N	N	N	N	N	N	N	N	N	N
Relaxation	N	N	N	N	N	N	N	N	N	N	N	N
Passivity	A	A	N	N	N	N	N	N	N	N	N	N
Narcosis	A	A	N	N	N	N	N	N	N	N	N	N
Ataxia	A	A	N	N	N	N	N	N	N	N	N	N
CNS STIMULANT												
Hyperactivity	A	A	A	A	A	A	A	A	A	A	A	A
Irritability	A	A	A	A	A	A	A	A	A	A	A	A
Stereotypy	A	A	A	A	A	A	A	A	A	A	A	A
Tremors	A	A	A	A	A	A	A	A	A	A	A	A
Convulsions	A	A	A	A	A	A	A	A	A	A	A	A
Straub tail	N	N	N	N	N	N	N	N	N	N	N	N
Analgesia	A	A	A	A	A	A	A	A	A	A	A	A
ANS ACTIVITY												
Ptosis	A	A	A	A	A	A	A	A	A	A	A	A
Exopthalmia	A	A	A	A	A	A	A	A	A	A	A	A
Urination	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N	N	N
Lacrimation	A	A	A	A	A	A	A	A	A	A	A	A

Behaviour of 6 animals in each group

Table 2: Effect of earthworm powder on bleeding and clotting time

Bleeding time				
S.No.	Group	0 minutes	60 minutes	120 minutes
1.	Control	*178± 0.8*	*180± 1.0*	*180± 1.2*
2.	Normal saline	*160± 1.5*	*168± 0.6*	*162± 0.5*
3.	Earthworm powder(100mg/kg)	*181± 1.5*	*198± 1.1*	*201± 1.2*
Clotting Time				
S.No.	Group	0 minutes	60 minutes	120 minutes
1.	Control	*125± 1.08*	*122± 0.6*	*120± 1.33*
2.	Normal saline	*128± 0.7*	*127± 0.8*	*125± 0.5*
3.	Earthworm powder(100mg/kg)	*130± 1.1*	*140± 0.8*	*165± 1.05*

Values are expressed as mean ± S.E.M. $p < 0.05$, (n=6).

Fig 1: Glycolipoprotein extract

Fig 2: Acute toxicity studies

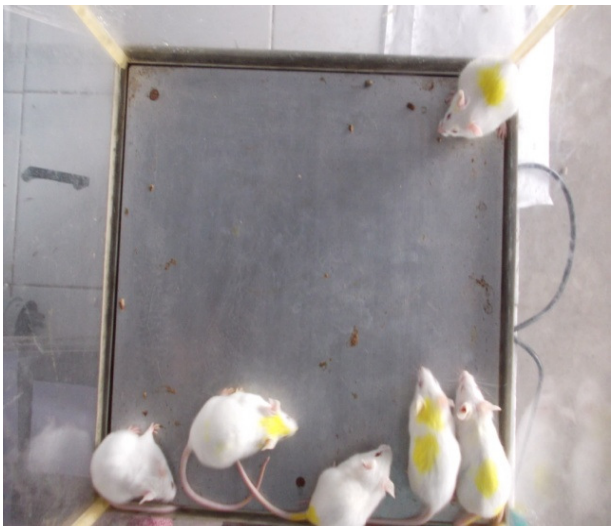

Fig 3: Initial Observation

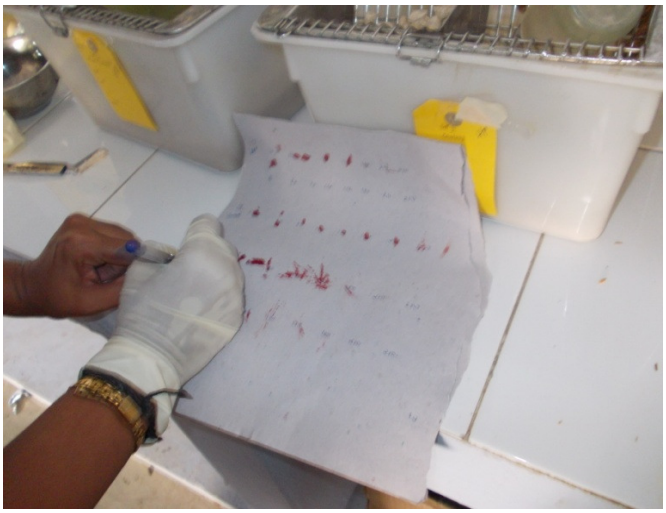

Fig 4: Calculation of bleeding time

CONCLUSIONS

Thus we conclude that the results motivate us for further investigations regarding clinical potentials of earthworm powder (glycolipoprotein) for the antithrombotic and fibrinolytic activities and its sensible pharmaceutical use in the field of human and veterinary medicine. Thus in future it could be a drug for cardiovascular diseases, although it still needs further investigation.

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