



PRE CLINICAL STUDIES FOR THE ANTI-INFLAMMATORY AND TOXICITY EVALUATION OF EARTHWORM

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ABSTRACT

Earthworms are precious and low cost source of numerous bioactive molecules. Recently, with the development of biomedicine, the research on pharmaceuticals derived from earthworms has been increasing. Acute Oral Toxicity Study of aqueous extract of earthworm was studied in *in vivo* model (albino mice) and it was revealed that there was no mortality or any signs of behavioral changes or toxicity observed after oral administration of earthworm aqueous extract. *In vitro* anti-inflammatory activities of earthworm samples were evaluated using albumin denaturation and membrane stabilization assays and the results indicated that the samples were effective in inhibiting heat induced albumin denaturation and heat induced hemolysis at different concentrations. The anti-inflammatory activity of samples in wistar rats was assessed by using various types of *in vivo* pharmacological screening methods such as Carrageenan induced rat paw edema and Croton oil induced inner ear edema. Studies have shown that administration of samples of earthworm in wistar rats which were induced of inflammation tends to normalize inflammation and the efficacy of coelomic fluid was similar to that of standard Indomethacin. From the results it was confirmed that earthworm samples possess potential anti-inflammatory activity.

KEYWORDS: hemolysis, edema, wistar rats, coelomic fluid, *Eudrilus eugeniae*.

1. INTRODUCTION

Traditional Chinese Medicines has been using earthworms for thousands of years. Although traditional medicines may be considered to be safe, some are known to be toxic at high doses and others may have potentially adverse effect after prolonged use. In order to establish the safety and efficiency of a new drug, pre-clinical toxicological studies are required in animals like mice, rat, guinea pig, dog, rabbit, monkey etc under various conditions of drug.

Inflammation is defined as the local response of living mammalian tissues to injury due to any agent. Inflammation involves two basic processes, early inflammatory response later followed by healing. There are two types of inflammation, Acute and Chronic.^[1] The clinically useful drugs against pain and inflammation exhibit many adverse effects and this leads to considerable interest in the search of safer drug for these conditions. Extracting and using biologically active compounds from earthworms have traditionally been practiced by indigenous people throughout the world.^[2]

Earthworm portions are usually used in the treatment of acute inflammatory conditions in some districts of South India.

Since the studies on medicinal properties of earthworm especially in the species *E. eugeniae* are limited, the present study investigates the anti-inflammatory and toxicity effect of coelomic fluid and aqueous extract of earthworm.

2. MATERIALS AND METHODS

2.1. Acute Oral Toxicity Study of Earthworm Extracts

Acute oral toxicity test was performed as per Organization for Economic Co-operation and Development (OECD) guidelines 423.^[3] The institutional ethical committee of KMCH College of Pharmacy, Coimbatore, Tamil Nadu, India, approved the protocol for these experiments under number KMCRET/Ph.D./06/2012-13. Experiments were performed using healthy young adult female Swiss albino mice, nulliparous, non-pregnant and weighing 25-

30 g (Fig. 1 and 2). The acute toxicity of Earthworm extract was studied as reported method with some modifications.^[4]



Fig. 1: Grouping of Mice

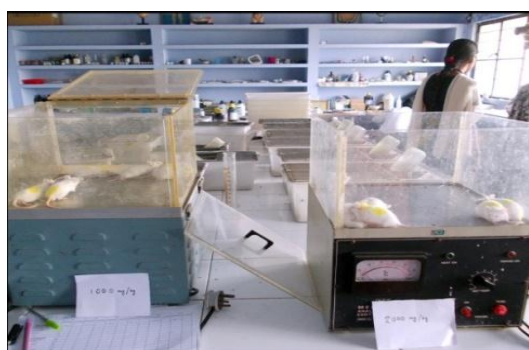


Fig. 2: *In vivo* Acute Toxicity

control group for determination of the percentage of inhibition of the paw edema. The efficacy of different earthworm samples were compared to standard positive anti-inflammatory drug, indomethacin.



Fig. 3: Grouping of Wistar Rats



Fig. 4: Inducing Inflammation

2.2. Anti-inflammatory studies

2.2.1. Assessment of *in vitro* anti-inflammatory activity

The anti-inflammatory activity of earthworm samples were studied by using Inhibition of albumin denaturation technique and Membrane Stabilization Assay according to^[5] followed with minor modifications.

2.2.2. Assessment of *In vivo* Anti-Inflammatory Studies

Male albino wistar rats weighing about 180-200 g were used for this study taking in to account international principles and local regulations concerning the care and use of laboratory animals.^[6] They were procured from KMCH Institute of Pharmacy, Coimbatore and maintained on the suitable nutritional and environmental condition throughout the experiment. The institutional ethical committee of KMCH College of Pharmacy, Coimbatore, Tamilnadu, India, approved the protocol for these experiments under number KMCRET/Ph.D./06/2012-13.

Acute Models of Inflammation

(i) Carrageenan-induced rat paw edema model

Anti-inflammatory activity was measured using carrageenan induced hind paw edema assay according to the method of^[7] with slight modifications (Fig. 3-7). The acute difference in edema volume was calculated in each control, test and standard group and compared with the



Fig. 5: Test substance administration



Fig. 6: Rat paw after Carrageenan injection



Fig. 7: Measuring the paw volume

(ii) Croton oil-induced ear edema

Croton oil-induced ear edema was performed according to the method described by^[8, 9] with slight modifications (Fig. 8). The edematous response induced by croton oil was assessed in terms of the increase in the weight of the right ear over that of the left ear. Inhibition percentage was calculated by comparison with the control group that only received the croton oil but none of the treatments.



Fig. 8: Application of croton oil in the rats ear

3. RESULTS**3.1. Acute Oral Toxicity Study of Earthworm Extracts**

The parameters observed before and after the administration of the test substance to the mice are presented in table 1. The present study conducted as per the OECD guidelines 423 revealed that there was no mortality or any signs of behavioral changes or toxicity observed after oral administration of extract up to the dose level of 2000 mg/kg body weight in mice. The oral LD₅₀ was indeterminable being in excess of 2000 mg/kg body weight. So, testing the extracts at a higher dose may not be necessary and the extracts were practically non-toxic.

Table 1: Acute Oral Toxicity Study

Gross Behavior	H		B		T		HB		BT		NM	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
<u>CNS Depression</u>	A	A	A	A	A	A	A	A	A	A	A	A
Hypo activity	A	A	A	A	A	A	A	A	A	A	A	A
Passivity	A	A	A	A	A	A	A	A	A	A	A	A
Relaxation	N	N	N	N	N	N	N	N	N	N	N	N
Narcosis	A	A	A	A	A	A	A	A	A	A	A	A
Ataxia	A	A	A	A	A	A	A	A	A	A	A	A
<u>CNS Stimulant</u>	A	A	A	A	A	A	A	A	A	A	A	A
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	P	P	P	P	P	P	P	P	P	P	P	P
<u>ANS Activity</u>	A	A	A	A	A	A	A	A	A	A	A	A
Ptoxis	A	A	A	A	A	A	A	A	A	A	A	A
Exophthalmia	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

H- Head, B- Body, T- Tail, HB- Head Body, BT- Body Tail, NM- No Marks

A- Absent, N- Normal, P- Present.

3.2. Anti-inflammatory studies

3.2.1. Assessment of *in vitro* anti-inflammatory activity

(i) Inhibition of albumin denaturation

As a part of the investigation of *in vitro* anti-inflammatory activity, the ability of earthworm extracts to inhibit protein denaturation was studied. The samples were effective in inhibiting heat induced albumin denaturation at different concentrations as shown in Fig 9. Earthworm coelomic fluid showed the maximum percentage of inhibition compared to earthworm aqueous extract.

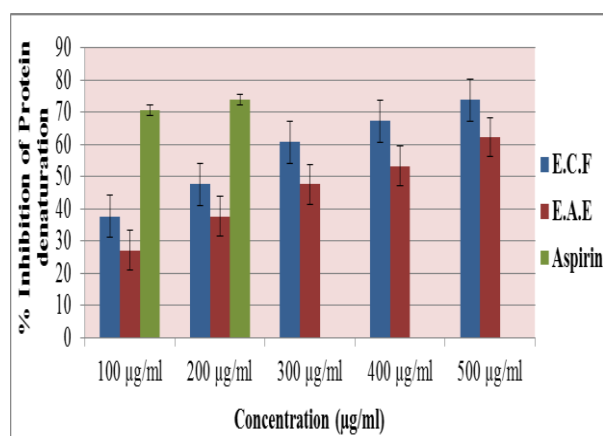


Fig 9: Effect of earthworm extracts on heat induced protein denaturation

E.C.F- Earthworm coelomic fluid, E.A.E- Earthworm aqueous extract.

Values are presented as the mean $\pm$ SEM (standard error of mean); n = 3 for all groups.

(ii) Membrane stabilization assay

The extract was effective in inhibiting the heat induced hemolysis at different concentrations. Test extracts (100 - 500 µg/ml) inhibited the heat induced hemolysis of RBCs to varying degree as shown in Fig 10. The results showed that earthworm coelomic fluid protect significantly ($p < 0.001$) the erythrocyte membrane against lysis induced by heat compared to earthworm aqueous extract. The anti-inflammatory activities of the extracts were dose dependent.

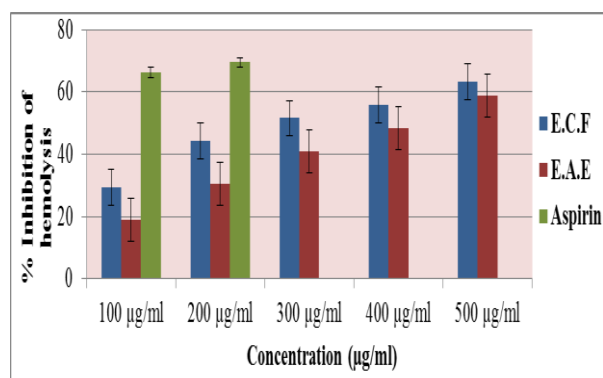


Fig 10: Effect of earthworm extracts on heat induced hemolysis

E.C.F- Earthworm coelomic fluid, E.A.E- Earthworm aqueous extract.

Values are presented as the mean $\pm$ SEM (standard error of mean); n = 3 for all groups.

3.2.2. Assessment of *In vivo* Anti-Inflammatory Studies

The anti-inflammatory activity was studied in Carrageenan Induced and Croton oil Induced Acute inflammation.

(i) Carrageenan Induced Rat Paw Edema (Acute Inflammation)

The effects of earthworm samples on carrageenan-induced edema in rats are shown in Fig 11. Significant percent increase in paw edema ($p < 0.001$) was observed in carrageenan treated control group when compared with drug treated groups. The aqueous extract of earthworm and coelomic fluid produced significant ($p < 0.001$) anti-inflammatory activity. The result indicates that coelomic fluid of earthworm possessed similar anti-inflammatory activity as that of indomethacin.

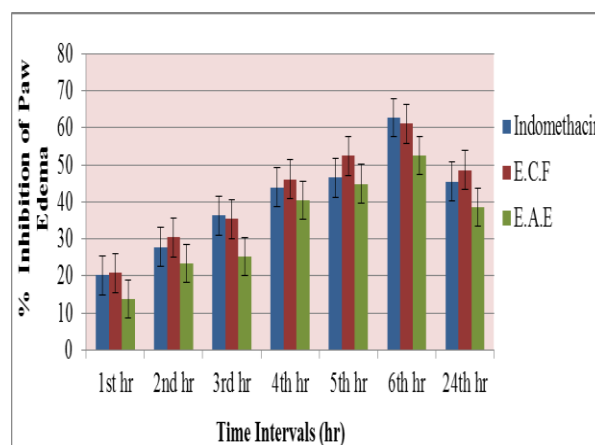


Fig 11: Carrageenan Induced Rat Paw Edema (Acute Inflammation)

E.C.F- Earthworm coelomic fluid, E.A.E- Earthworm aqueous extract.

Values are presented as the mean $\pm$ SEM (standard error of mean); n = 3 for all groups.

(ii) Croton Oil Induced Inner Ear Edema

The effects of orally administered samples and Indomethacin on croton-oil-induced inner ear edema are shown in the Fig 12. In the croton oil induced ear test, the inflammatory response was also quantified by measuring the ear plug weight (EPW). The biopsied ear tissue were weighed at the end and showed a great difference between the two ears (left and right) in control group, whereas the difference was less in the case of standard and treatment groups. Thus the results indicated that coelomic fluid and aqueous extract of earthworm showed significant reduction of ear edema by croton-oil in wistar rats.

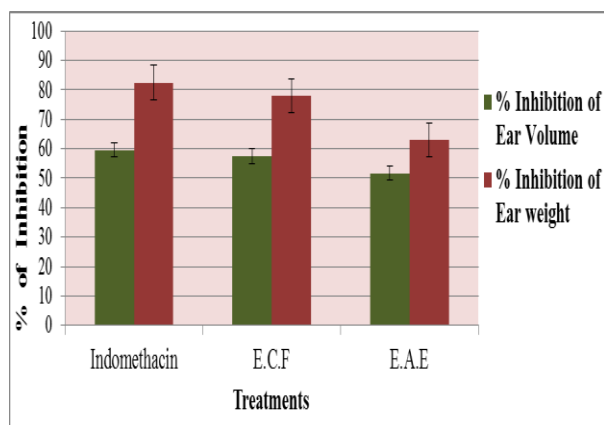


Fig 12: Croton Oil Induced Inner Ear Edema (Acute Inflammation)

E.C.F- Earthworm coelomic fluid, E.A.E- Earthworm aqueous extract.

Values are presented as the mean $\pm$ SEM (standard error of mean); $n = 3$ for all groups.

4. DISCUSSION

The present study conducted as per the OECD guidelines 423 revealed that there was no mortality or any signs of behavioral changes or toxicity observed after oral administration of earthworm aqueous extract up to the dose level of 2000 mg/kg body weight in mice. The oral LD₅₀ was indeterminable being in excess of 2000 mg/kg body weight. So, testing the extract at a higher dose may not be necessary and the extract was practically non-toxic.^[10] evaluated the acute toxicity effects of earthworm powder (EWP) obtained from *E. eugeniae* on wistar male rats. They observed that 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. According to some literature guidelines, materials or substances with a p.o. LD₅₀ of 500–5000 mg/kg are regarded as virtually safe.^[11]

Most biological proteins lose their biological function when denatured. Denaturation of protein is a well known cause of inflammation.^[12] As a part of the investigation of *in vitro* anti-inflammatory activity, the ability of earthworm extracts to inhibit protein denaturation was studied. Earthworm coelomic fluid showed the maximum percentage of inhibition, 73% at a concentration 500 μ g/ml.^[12] reported that *Enicostemma axillare* methanol extract (EAME) was effective in inhibiting heat induced albumin denaturation. Maximum inhibition of 71% was observed at 500 μ g/ml. Aspirin, a standard anti-inflammatory drug showed the maximum inhibition 68% at the concentration of 100 μ g/ml compared with control.

Stabilization of the RBCs membrane was studied to further establish the mechanism of anti-inflammatory action of earthworm extracts. The results showed that earthworm extracts at a concentration of 500 μ g/ml protect significantly ($p < 0.001$) the erythrocyte membrane against lysis induced by heat. The anti-inflammatory activities of the extracts were dose dependent.^[12]

reported that *Enicostemma axillare* methanol extract (EAME) at concentration 400 and 500 μ g/ml protect significantly ($p < 0.05$) the erythrocyte membrane against lysis induced by heat. The erythrocyte membrane is analogous to the lysosomal membrane^[13,14] and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The extra cellular activity of lysosomal enzymes are said to be related to acute or chronic inflammation. The non steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane.^[15]

In the present study, the anti-inflammatory activity of samples in wistar rats was assessed by using *in vivo* pharmacological screening methods such as Carrageenan Induced rat paw edema and Croton oil Induced inner ear edema. In Carrageenan Induced rat paw edema, the aqueous extract of earthworm and coelomic fluid produced significant ($p < 0.001$) anti-inflammatory activity and reduced the paw volume by 52% and 61% respectively at 6th hour after carrageenan administration. Indomethacin at 10 mg/kg inhibited the edema volume by 62% at the 6th hour post induction. The result indicates that coelomic fluid of earthworm possessed similar anti-inflammatory activity as that of indomethacin. Through the results obtained in the croton oil-induced ear edema test, the anti-inflammatory effects of earthworm samples were verified when compared with the control group. Thus the results indicated that coelomic fluid (50 mg/kg) and aqueous extract of the earthworm (100 mg/kg) showed significant reduction of ear edema caused by croton-oil in wistar rats at 77 and 62% respectively.

There are numerous studies on the anti-inflammatory properties of various plant extracts. But only a few studies have been made on the same from animal origin.^[16] reported that earthworm extract shows more than 70% inhibition of oedema, 3 hrs after carrageenan injection and hence it is likely, that its anti-inflammatory action is similar to that of non-steroidal anti-inflammatory drugs. He also reported that TEP produced a dose dependent decrease in paw volume up to a dose of 160 mg/kg, beyond which, anti-inflammatory effect was present, but not significantly greater than that of 160 mg/kg. According to his report TEP in doses of 80 mg/kg and 160 mg/kg exhibited greater anti-inflammatory effect than hydrocortisone but less than that of phenylbutazone.^[17] established that, the petroleum ether fraction of total earthworm paste, has better anti-inflammatory activity in albino rat and they found that 160 mg/kg total earthworm paste functions similarly to that of aspirin in carrageenan-induced oedema.^[18] reported that the carageenan induced acute phase rat hind paw edema volume and the turpentine induced chronic

phase granuloma pouch weight and the volume of fluid were reduced significantly due to the administration of aspirin. However, the administration of earthworm paste at a dose of 80 mg/kg was found to reduce all the above parameters brought to near normalcy.

Compounds of biological origin are known to possess minimal residual effect. The present study has confirmed the anti-inflammatory activity of coelomic fluid and aqueous extract of earthworm *E. eugeniae* in the *in vivo* models of inflammation, which may be mediated through inhibition of cell mediators such as bradykinin and prostaglandins. Administration of earthworm samples in to wistar rats which were induced of inflammation, shows significant anti-inflammatory activity and restore the levels of antioxidants. Further studies are needed to evaluate the actual principal compounds present in the earthworm, which act as a therapeutic agent.

5. CONCLUSION

Folk and traditional medicine is a promising field for discovering new therapeutics. In order to meet the increasing demand of natural medicines in recent years, bioactive components with medicinal values from earthworms have already provoked increased interest. The pharmaceutical effects of earthworm *Eudrilus eugeniae* was found to be productive, as evidenced from the observations of present investigations. The observations and results of the present study have opened up a new outlook and paved the way to explore the significance of earthworms. This study clearly indicates that earthworms can be used not only in environmental monitoring but also in the acquisition of novel molecules for human therapeutic purposes.

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