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ANTI-INFLAMMATORY EFFECT OF EXTRACT OF EARTHWORM-*EUTYPHOEUS GAMMIEI* FROM TRIPURA, NORTH EASTERN INDIA

Debnath Madhusudan[1], Gosh Ranjib[2], Bhattacharjee Deepjyoti[3], Bhattacharjee Surajit[4] and Sil Samir Kumar[5*]

[1]Department of Human Physiology, Tripura University,
[2]MBBS, MD, Pharmacology, Tripura Medical College & Dr. B.R. Ambedkar Memorial Teaching Hospital,
[3]Research Scholar, Dept. of Human Physiology, Tripura University,
[4]Departmental of Molecular biology and Bio informatics, Tripura University, Suryamaninagar,
[5*]Departmental of Human Physiology, Tripura University, Suryamaninagar-799022, Tripura, India

***Corresponding Author Email: madhudn@rediffmail.com**

ABSTRACT

Earthworms have been used in medicine for various remedies. In the present investigation, different solvent extracts of an earthworm, E. gammiei were prepared and anti-inflammatory activities of these extracts were determined. The petroleum ether fraction possessed maximum anti-inflammatory activity in carrageen induced albino rats in comparison to 90% ethanol and 0.2M phosphate buffer (pH, 7.0) extracts. The paw volume was determined and was compared with that of aspirin, a standard anti-inflammatory drug. The results indicate that petroleum ether fraction of earthworm extract possessed near about similar anti-inflammatory activity as that of aspirin.

KEY WORDS

E. gammiei, Tripura, anti-inflammatory activity, petroleum ether, ethanol extract, phosphate buffer saline.

INTRODUCTION:

For several years earthworms have been widely used in china, Japan, Indonesia, and the Far East to treat various chronic diseases. Intensive exploration has been made to reveal the use of earthworm as anti-microbial (Popovic *et al*., 2005), anti-inflammatory, anti-pyretic (Balamurugon *et al*., 2009 and Ranganathan *et al*., 2009), and anti-cancer (Chen *et al.,* 2007) agents. Earthworm contains many compounds with potential medicinal properties & have been administered to treat inflammatory, hematological oxidative & nerve disease (Cooper *et al.,* 2007; Chen *et al.,* 2010; Liu *et al.,* 2013). It has been reported that medicinal activities of earthworm may vary depending on the species & living environment of that organism.

Earthworms have been used in medicine for various remedies since 1340 AD (Stephenson J, 1930). Earthworm has been recognized in oriental medicine as anti-inflammatory, analgesic and antipyretic agent (Noda N *et al.,* 1992). It shows anticancer effect by preventing excess glucose uptake (Nagasawa H *et al.,*1991). Microorganisms are known to play a major role in soil characteristics, invertebrates are believed to act as regulators of antimicrobial activity. Earthworm surface excreta were found to have potent antimicrobial activity (Oleynik AS *et al.,* 2008). It is also having anticoagulatory or fibrinolytic activity which results in the facilitation of blood circulation (Wang JD *et al.,* 1989). The earthworm has been suspected to contain proteases which dissolve the filbrin clots or anticoagulants which selectively interfere with the

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intrinsic pathway of blood coagulation cascade (Mann KG *et al.,*1991) Medicine properties of earthworm have been described (Bristow HS et al., 1932)

Earthworms have largely been used internally and externally as powerful (Vohora *et al.,*1978). Anti-inflammatory activity of earthworm extracts was studied (Ismail SA *et al.,* 1992) The anti-inflammatory and antipyretic activities of biologically active extract isolated form whole earthworm, *Lampito mauritii* were determined. Antimicrobial potency of *Eutyphoeus gammiei* extracts on Bacteria were studied (Shobha SV and Kale, 2007). Antitumor activities of earthworm fibrinolytic enzyme on human hepatoma cells were studied (Hong C, 2007). The species selected for study was *Eutyphoeus gammiei.* This species is native of Africa and is having good reproduction and maturation capability. In the present investigation, different solvents were used on the basis of increasing polarity such as petroleum ether, 95% ethanol and 0.2 M (pH, 7.0) phosphate buffer to prepare earthworm extracts in order to assess their anti-inflammatory activity.

In this work endeavor has been made to explore the anti-inflammatory properties of earthworm species, *E. gammiei* collected from the state of Tripura, North-East India.

MATERIALS AND METHODOLOGY:

Animal Selection:

For accomplishment of the entire experiment male Swiss albino rat (120-150 gm b.w.) were procured from an authorized animal supplier from Kolkata. According to the guideline of CPCSEA, the animals were maintained in suitable laboratory condition for one week before the commencement of treatment. The Institutional Animal Ethical Committee (IAEC) approved the proposed Ph. D work [Approval no TU/IAEC/2016/XIII/I dated 30th August 2016].

Animal diet and maintenance:

Standard animal food pellet containing 18% milk protein was provided to all of the animals during the experimental tenure. Albino rat supplied with purified drinking water throughout the treatment schedule. They were kept in the treatment room with sustaining 22°C to 25°C temperature and humidity (50%) with alternate light and dark coverage for 12 hours. Special attention was given regarding regular cleanliness of the animal house and day to day activities of the treated animals throughout the treatment schedule.

Animal treatment:

Treatment plan for dose dependent study:

To perform the dose dependent study, Swiss albino rat (N=20) were procured from authentic animal supplier stated earlier. Initially, the animals were distributed into two distinct groups having equal mediocre body weight (120-150g); the control Cr (VI)-treated groups. Further, the albino rat of the treated group were subdivided into four separate groups for the dose dependent study. Each group consisted of four numbers (n=4) of rat which were allowed to proceed through the following treatment schedule.

Selection of a specific dose:

From the dose-dependent study a specific dose of pet ether was selected at which significant metabolic toxicity occurred without any casualty. That dose appeared to be 10 mg per kg body weight per day for 30 days.

Experimental design with that selected dose:

Healthy albino rat of body weight ranging from 120-150gm were chosen for the current study and equally distributed into control group and Cr (VI) treated group, each group having four numbers of animals.

Preparation of tissue homogenate:

The tissue homogenate was made in 0.1 M phosphate buffer solution (pH 7.4) and also in 0.25 M sucrose solution separately according to the biochemical protocol. Specific amount of tissue was weighted to prepare 5% (w/v) and 10% (w/v) tissue homogenate as needed for various analytical procedures by using Potter Elvenjem glass homogenizer and stored at-20°C unit analyses.

Sample collection and identification:

Adult earthworm *Eutyphoeus gammiei* was collected by hand sorting and digging method by spade from Agartala, Tripura at early morning. The sample was first identified by Prof P.S.Choudhuri, Earthworm Research Laboratory, Department of Zoology, Tripura University. The sample specimen also submitted to ZSI, Kolkata for authentication (voucher number- An 5649/1).

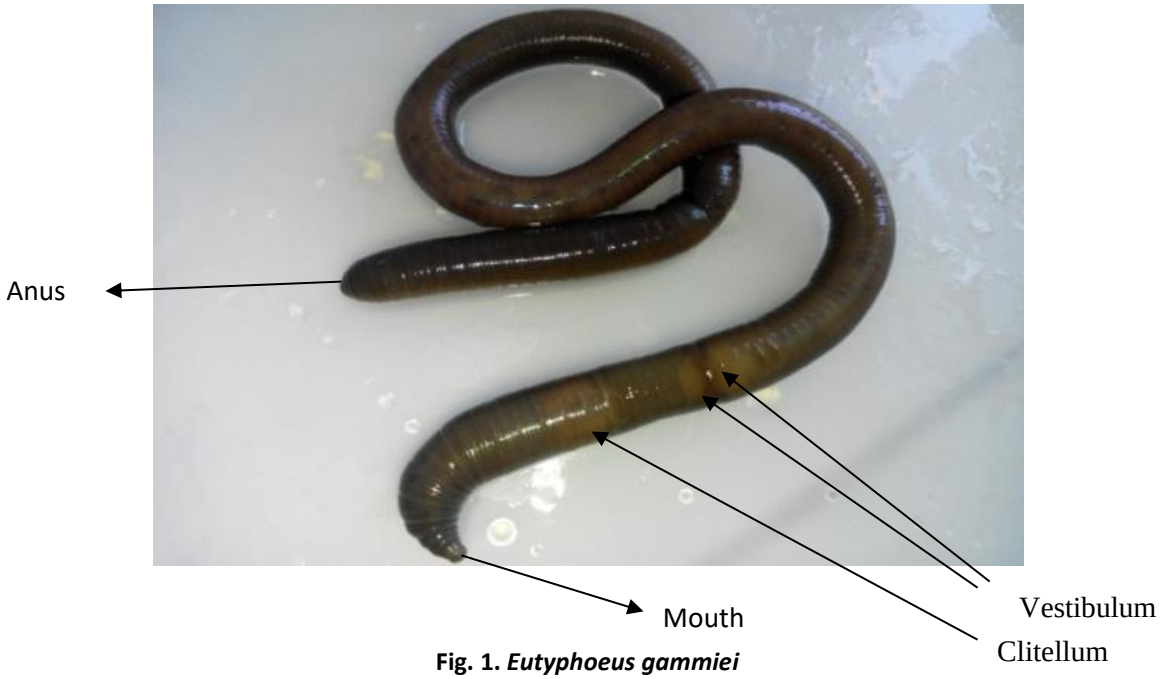

Fig. 1. *Eutyphoeus gammiei*

Preparation of crude earthworm extract:

Earthworm *Eutyphoeus gammiei* were washed with running tap water and then fed with wet floating paper for 18-20 hrs to clear their gut. The gut cleared worms were again washed with distilled water. Then worms were dried at 40°C temperature. To get the crude extract, the dried sample was extracted with 95% ethyl alcohol and petroleum ether. The crude Ethanol and petroleum ether extract of *Eutyphoeus gammiei* obtained were diluted in PBS (phosphate buffer solution) for evaluation of anti-Inflammatory activities.

Determination of anti-inflammatory activity:

Animal model was used for evaluating the anti-inflammatory activity. Healthy male albino rats weighing to 100-150g were selected for the study. The animals were divided into five groups of three rats each. Pedal edema was produced by sub planter injection of 0.1 ml carrageenan (1g%) in left front paw. Paw volumes were measured before and after 3 h of the injection to record the degree of inflammation. Three hours following the injection, the first group (control) was offered distilled water intraperitoneally. The second group was injected with PBS. The third group was injected with Ethanol extract. The fourth and fifth groups were injected with petroleum ether and aspirin (160 mg/kg body weight fraction and 95%) respectively. This dosage of 160 mg/kg body weight has been standardized in previous studies (Ismail et al., 1992; Balamuragan et al., 2008). Paw volumes were measured again after 1h, 2h and 3h of treatment following anesthetization of the rats. The experiment was done in triplicates and the efficacy of different earthworm extracts was compared to standard positive anti-inflammatory drug, aspirin.

Qualitative screening of extracts for bioactive molecules:

Screening of Ethanol and petroleum ether extracts of *Eutyphoeus gammiei* was carried out based on standard protocols.

Detection of alkaloids (Wagner's test): Extract was dissolved individually in dilute Hydrochloric acid and filtered. Filtrate was treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Detection of Phenolics (Ferric Chloride test): Extract was treated with 3-4 drops of ferric chloride solution. Formation of bluish black indicates the presence of phenols.

Detection of Flavonoids (Alkaline reagent test): 2 ml of extract was treated with few drops of 20% NaOH solution. Formation of intense yellow colour which becomes colourless on addition of dilute HCl in the presence of Flavonoids in the extract.

Detection of Terpenoids (Salkowski test): To 0.5g each of the extract was added 2ml of chloroform. Concentrated H_2SO_4 (3ml) was carefully added to form

a layer. A reddish brown colouration of the interface indicates the presence of Terpenoids.

Detection of Quinones (Con. HCL Test): 2 ml of extract was treated with concentrated HCl. Formation of yellow precipitate or colouration indicate the presence of Quinones in the extract.

Detection of Steroids (Keller-Killani Test): 2-5 ml of extract was added to 2.5 ml glacial acetic acid with 1 ml of 5% ferric chloride treated with 2.5 ml of contracted H_2SO_4. Appearance of a brown colour ring in the juncture if the two liquid indicates the presence of Steroid in the extract.

RESULTS AND DISCUSSION:

It was observed that carageenan induced acute phase edema in the front paw and the volume of fluid was reduced significantly due to administration of *Eutyphoeus gammiei*, extract (Table.3.10 fig.2.15). However, pet ether extract exhibited better result compared to that of 95% ethanol extract. Result of petroleum ether was comparable to the result of positive control, aspirin. At 160 mg/kg dose, petroleum ether extract reduced the volume to normalcy after 3 hr as was also observed in case of positive control.

Table.3.10. Anti-inflammatory activity of various fractions of *Eutyphoeus gammiei* extract

Experiment	Normal	3hr After Carrageenan injection	1 hr after treatment	2 hr after treatment	3 hr after treatment
Control (H2O)	2.50±0.00	5.47±0.00	4.33±0.06	4.57±0.07	4.60±0.19
PBS	3.00±0.17	5.90±0.03	3.83±0.09	3.83±0.12	3.83±0.06
Ethanol extract	2.63±0.03	6.10±0.06	3.10±0.09	3.33±0.06	3.10±0.09
Pet ether extract	2.93±0.15	5.56±0.01	2.83±0.09	2.80±0.15	2.80±0.03
Aspiran	2.70±0.03	5.70±0.00	3.24±0.06	2.51±0.02	2.50±0.06

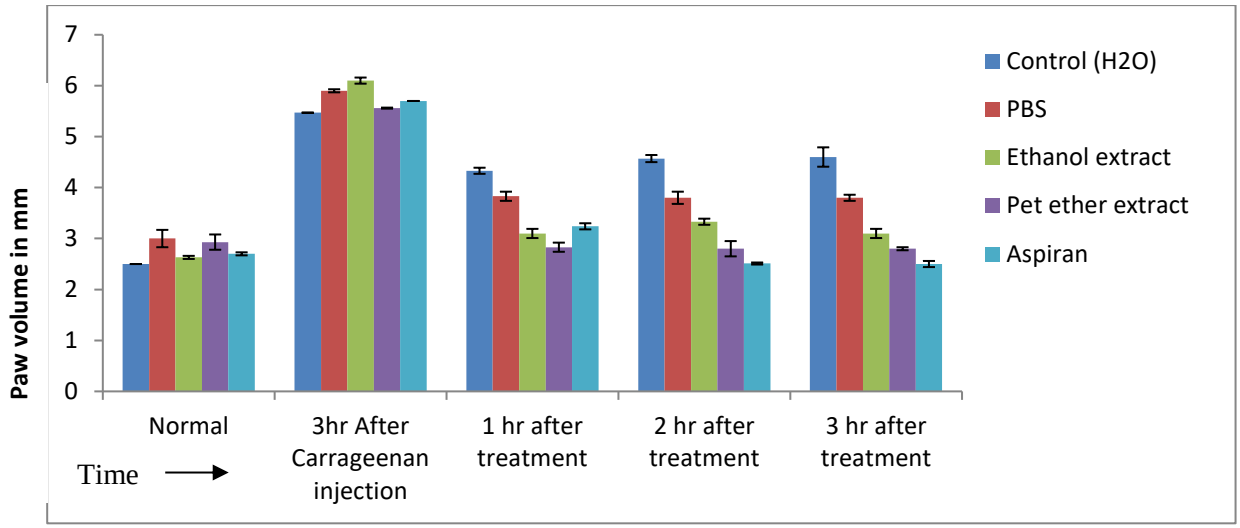

Fig.2.15. Anti-inflammatory effect of various fraction of *Eutyphoeus gammiei* extract.

Qualitative analysis of extract for bioactive molecules:

Qualitative studies of earthworm extract was performed on its alcohol and petroleum ether extracts to identify its alkaloids, phenolic compounds, flavonoids, terpenoid, steroid, tannin, Quinones **by** using suitable chemicals and reagents (Table 3.11). The color intensity was shown as '+', '++', '+++' and '++++' for low/slight, moderate, good and high means positive tests respectively and '-' for no color change meaning negative test. Phenolic compounds were absent in both the extracts. However, concentration of Terpenoids and steroids were found is more in concentration in petroleum ether extract.

Table.3.11. Qualitative analysis of E. *gammiei* extract for bioactive molecules

Chemical constituents	Tests	Ethanolic of extract of Earthworm	Pet ether extract of Earthworm
Alkaloid	Wagners test	+	+
Phenolics	Ferric Chloride test	-	-
Flavonoids	Alkaline reagent (NaOH)	++	++
Terpenoids	Conc. H_2SO_4 + Chloroform test	+++	+++++
Quinones	Con. HCL Test	+	+
Steroid	Acetic anhydrous Test	++	+++++

-, Negative; +, Slight; ++, Good; +++, Moderate; ++++, Strong

Anti-inflammatory activity of different species of earthworm have been reported (Ismail *et al.,* 1992; Balamurugan *et al.,* 2008). In this study anti-inflammatory activity of *E. gammiei* was evaluated in carrageenan induced animal model. Petroleum ether extract and ethanol extract of *E. gammiei* were tested for anti-inflammatory activity according to Mathur *et al.,* (2011). Petroleum ether extract was found to be more potent in respect to ethanol extract (Table 3.10, Fig. 2.15). Mathur *et al.,* (2011) also observed similar response in case of *Eudrilus eugeniae* species of earthworm. Presence of more amount of sterol and terpenoids in petroleum ether, as evident from qualitative analysis for bioactive molecules (Table 3.11), could be the contributory factor for higher anti-inflammatory activity of petroleum extract.

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CONFLICT OF INTEREST:

The author declared no conflict interest/among themselves.

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***Corresponding Author:**
Sil Samir Kumar*
Email: **madhudn@rediffmail.com**