International Journal of Advanced Research and Development
ISSN: 2455-4030
Impact Factor: RJIF 5.24
www.advancedjournal.com
Volume 3; Issue 1; January 2018; Page No. 283-292

Evaluation of anti-inflammatory activity of earthworm (*Eudrilus eugeniae*) extract through animal models

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Abstract
In the pursuit of new anti-inflammatory drug that should be less toxic and more effective, scientists are working new and alternative approaches to fight inflammatory diseases hence this study was planned with earthworm *Eudrilus eugeniae* to pave a way in the quest of new anti-inflammatory drug. Two *in vivo* experiments (carageenan induced paw oedema and cotton pellet induced granuloma pouch) were conducted on experimental rats to demonstrate anti-inflammatory activity of EW extracts. Both of these treatments were found to cause acute and chronic inflammatory reactions. The inflammation was found to be significantly suppressed by pre-treatment with EW extracts. Above anti-inflammatory experiments were additionally used for biochemical demonstration of parameters of oxidative stress markers like reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS), super oxide dismutase (SOD) and catalase activities. The results have demonstrated that the levels of oxidative stress markers were significantly higher in control than experimental (Indomethecin and EW extract treated) animals indicating significant oxidative stress removing activity. The level of cytokines TNF-α and IL-10 were used to demonstrate as marker of inflammatory and anti-inflammatory conditions in both acute and chronic model of inflammation. Declining levels of TNF-α and increasing trend of IL-10 indicate anti-inflammatory effect of EW extract.

Keywords: earthworm, *Eudrilus eugeniae*, anti-inflammatory, anti-oxidant, cytokine, indomethacin

1. Introduction
Inflammation is a primary physiologic defense mechanism of the body that helps to protect it against infection, burn, toxic chemicals, allergens or other noxious stimuli and characterized by pain, redness, swelling, and sometimes loss of sensation. An uncontrolled and persistent inflammation may act as causative factor for many of the chronic illnesses (Sosa *et al.*, 2002) [35]. A number of anti-inflammatory synthetic drugs are available, which are effective but may possess several side effects (Bennett and Brown, 2003) [5] and therefore, it is imperative that these synthetic drugs can be replaced with compounds that are equally efficacious, but less toxic and comparatively free from side effects. For achieving this goal researches are going on with full pace around the world to discover newer and less harmful or harmless medicines.

Earthworms are an integral component of complex soil ecosystem to play major role in nutrient recycling. Since Darwin, their tremendous role in the aeration, formation, structure, texture, water seepage and fertility of the soil has been greatly respected. The contribution and very diverse roles of earthworms in human life has been poorly recognized in modern human civilization. In fact earthworms are proving themselves to be a great "biological resource" for mankind as a waste decomposer, bio-fertilizer manufacturer, land reclaimer, protein producer, as drug source etc. Medicinal value of earthworms is known since thousand years as it is evident from history of ancient medicine from various parts of the world. Earthworm powder was used to treat jaundice (Stephenson, 1930) [36]. According to historical records earthworms are useful in curing rheumatism also (Reynolds and Reynolds, 1972) [30]. From 1700s, earthworms were commonly used in European medical practice and regarded as highly diuretic, diaphoretic and analgesic (Dale, 1693; Rota, 2011) [8, 32]. According to ancient chinese medical book "Compendium of Materia Medica" (1552-1593), Shen (2010) [33] described that earthworm can be used for medicinal purpose in three different ways: (1) As powder by grinding the dry earthworms (2) As decoction and (3) As ash. A strong fibrinolytic enzyme complex was isolated and purified from earthworm, *Lumbricus rubellus* (Mihara *et al.*, 1983, 1989, 1991, 1992, 1993; Lu *et al.*, 1988; Nakajima *et al.*, 1993, 2000; Cong *et al.*, 2000; Lin *et al.*, 2000; Cho *et al.*, 2004) [21-26, 27, 7, 6]. Therapeutic and preventive effects, of the drug, for thrombosis related diseases have been confirmed clinically (Jin *et al.*, 2000) [16]. A lot of research work has been carried out in Vermicomposting Centre of Department of Zoology, Jiwaji University, Gwalior. Four species of epigeic earthworms (*Eudrilus eugeniae*, *Eisenia fetida*, *Perionyx excavates and Perionyx cressiseptatus*) are available in the Centre for Vermicomposting of different waste material including cattle dung, animal house waste, pea waste, paper waste, kitchen and food waste, temple waste, sewage sludge, garden waste. On the basis of research work, it can be concluded that out of the four species, the performance of *Eudrilus eugeniae* is the best. Only few studies have been

done by Indian workers dealing with medicinal aspects of earthworms.

Therefore anti-inflammatory activity of extract of *E. eugeniae* was investigated with the help of two models *i.e.*, acute model (carrageenan induced rat paw oedema) and chronic model (cotton pellet induced granuloma pouch).

2. Materials and Methods

Procurement and Maintenance of Animals

Adult Wistar rats were procured from Defence Research and Development Establishment (DRDE), Gwalior, India. They were maintained in animal house facility of School of Studies in Zoology of Jiwaji University, Gwalior under standard conditions (25 ± 2^0 C, 55-60% RH and 12:12 hour light and dark cycling) and they were provided with standard laboratory rat feed pellets (Ashirvad Industries, Chandigarh) and water *ad libitum.* All experimental protocols and procedures were in accordance of institutional regulations and national criteria for animal experimentation and due approval was taken from the Institution Animal Ethics Committee.

Culture and Maintenance of *Eudrilus eugeniae*

Adult earthworms, *Eudrilus eugeniae* (Annelida: Oligochaeta: Lumbricidae) were procured from the mother culture maintained in a large sized vermi-tank at Vermicomposting Center, located in Charak Udyan, Jiwaji University, Gwalior, (M. P.), India (Figs. 1, 2, 3). The earthworm culture was maintained in a mixture of plant leaves and cattle dung (standard medium).

Preparation of Earthworm Extract

Earthworm extract was prepared according to Hrzenjak *et al.* (1992) [14] with some modifications. Sexually mature clitellate worms (1.345 gm/worm approx.), collected from the stock culture, were thoroughly washed under running tap water to remove dung, debris etc. attached on their body surface. Then the worms were kept in 0.65% NaCl solution, at room temperature for two hours for removal of cast from the body. The solution of NaCl was changed after every hour. After two hours, NaCl solution was discarded and the animals were transferred in a glass tray. Tray was covered tightly by a cotton cloth through which air can penetrate. Earthworms were then left overnight for maximum release of cast from their body. Worms were then minced with a pair of scissors. Three grams of minced earthworm tissue was weighed and was mixed with 40 ml of chloroform-methanol (V/V) solution. The mixture was then homogenized and kept at 4ºC overnight. The very next day, 16 ml of distilled water was added to the homogenate. It was mixed and centrifuged at 5000 RPM for 10 min. to obtain three clearly differentiated layers. The upper water - methanol layer was pipetted out and evaporated at 37^0C in a water bath until brown colored paste was obtained. It was stored in Eppendorff (micro-centrifuge) tubes and kept at -20º C for further use.

Anti-inflammatory Activity

This activity was evaluated by two models

a) Acute model study
b) Chronic model study

A) Acute model study or carrageenan induced rat paw oedema model (Winter *et al.*, 1962) [40]

Principle: This model is based on the principle of release of various inflammatory mediators by carrageenan. Oedema formation due to carrageenan in the rat paw is biphasic event. The initial phase is attributed to the release of histamine and serotonin. The second phase of oedema is due to the release of prostaglandins, protease and lysosome. Subcutaneous injection of carrageenan into the rat paw produces inflammation resulting from plasma extravasation, increased tissue water and plasma protein exudation along with neutrophil extravasation, all due to the metabolism of arachidonic acid. The first phase begins immediately after injection of carrageenan and diminishes in two hours. The second phase begins at the end of first phase and remains through third hour to five or six hours.

Procedure

Anti-inflammatory activity of earthworm extract was demonstrated by carrageenan induced paw oedema method of Winter *et al.* (1962) [40] in male albino Wistar rats. In this method experimental animals were injected with 0.1 ml of 1% solution of carrageenan into sub-planter side of left hind paw. Left paw was marked with permanent marker at the level of lateral malleolus. Carrageenan injection induces oedema of the paw. Basal paw volume was measured using plethysmograph (Fig. 4) at 0 hour (immediately after injection of carrageenan), then at hourly interval up to 6 hours. The paw was immersed till the level of lateral malleolus and the volume displacement was measured. Extent of swelling is directly proportional to the volume displaced that is a measure of degree of inflammation. In the present study male albino Wistar rats (180-200 gm) were starved overnight and next day they were divided into six groups (n = 6 in each group) and they were treated in following manner:

- Group I: experimental control, inflammation induced, received no treatment
- Group II: negative control, inflammation induced, received normal saline (1ml/Kg body weight)
- Group III: positive control, inflammation induced, received Indomethecin (10mg/kg body weight)
- Group IV: test group, inflammation induced, received earthworm extract (150mg/kg body weight)
- Group V: test group, inflammation induced, received earthworm extract (200mg/kg body weight)
- Group VI: test group, inflammation induced, received earthworm extract (250mg/kg body weight)

All the treatments including earthworm extracts, standard drug and normal saline were administered in respective groups one hour before the subcutaneous challenge by the injection of carrageenan.

Calculation: The increase in paw volume is calculated as percentage compared with the basal volume. The difference of average values of treated animals and control group was calculated from their respective basal volume for each time interval and evaluated statistically. The per cent inhibition was calculated using the formula as follows:

$$\% \text{ Oedema inhibition} = 1 - \frac{Vt}{Vc} \times 100$$

Where
Vt= Volume of paw of treated group
Vc= Volume of paw of control group

B) Chronic Model Study or cotton pellet induced granuloma pouch method (Goldstein *et al.*, 1976) [13]

Principle: The subcutaneous implantation of foreign bodies (compressed cotton pellets) induces formation of granuloma pouch. The foreign bodies are invaded by giant cells and undifferentiated connective tissue so as to make a gruanuloma pouch in several days. The amount of deposited cells and connective tissue can be measured by weighing dried pellets after removal of granuloma. More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenan.

Procedure

Anti-inflammatory activity was carried out by using cotton pellet-induced granuloma pouch model by the method of Goldstein *et al.* (1976) [13]. Male albino Wistar rats (180-200 gm) were starved overnight and next day they were divided into six groups (n =6) which were treated in the following way:

- Group I- experimental control, inflammation induced, received no treatment
- Group II- negative control, inflammation induced, received normal saline (1ml/Kg body weight)
- Group III- positive control, inflammation induced, received Indomethecin (10mg/ kg body weight)
- Group IV- test group, inflammation induced, received earthworm extract (150mg/ kg body weight)
- Group V- test group, inflammation induced, received earthworm extract (200mg/ kg body weight)
- Group VI- test group, inflammation induced, received earthworm extract (250mg/ kg body weight)

All the treatments including earthworm extracts, standard drug and negative control (normal saline) were orally administered in respective groups one hour before the anesthetization of animals by ether. After anesthetization, skin of the groin area of animals was shaved and disinfected with 70% ethanol. An incision was made and subcutaneous tunnel was formed by a blunted forceps and sterilized cotton pellet (20 mg), impregnated with carrageenan, was inserted in the tunnel. The incision was sutured by sterile suturing needle using sterilized thread. All the animal groups were maintained with due care and supply of food and water for next 7 days. On 8th day, animals were again anesthetized by ether and wet cotton pellet were carefully removed surgically. Pellets were separated from extraneous tissues and dried at 60^{o}C overnight to get constant mass. The net dry weight of the pellet were determined after subtracting the initial weight of cotton pellet and compared with control.

Calculation

The per cent inhibition of inflammation was calculated by following formula

$$\% \text{ inflammation inhibition} = 1 - \frac{Vt}{Vc} \times 100$$

Where
Vt = Dry pellet weight of treated group
Vc = Dry pellet weight of control group

Evaluation of Oxidative Stress Markers

A number of biochemical markers, reduced Glutathione (GSH) (Ellman, 1959) [11], superoxide dismutase (SOD) (Winterbourn *et al.,* 1975) [41], thiobarbituric acid reactive substance (TBARS) (Ohkawa *et al.*, 1979) and catalase (CAT) (Sinha, 1971) [34] have been identified to represent antioxidant activities. Estimation of these markers was carried out in blood samples withdrawn from retro orbital vein of rats and used in both types of anti-inflammatory activities, described above. The blood was collected in 3.8% Sodium Citrate (anticoagulant) in 9:1 ratio.

Sample Collection for Oxidative Stress Markers

A part of citrated whole blood sample (about 0.2 ml) was used for estimation of GSH and remaining sample was used for preparation of haemolysate for estimation of CAT, SOD and TBARS. All estimations were carried out immediately after collecting the blood samples. In addition to these parameters, the haemolysate samples were also subjected for protein estimation according to Folin-Lowry Method (Lowry *et al.*, 1951) [18].

Measurement of Cytokines

Concentrations of TNF-α and IL-10 were analyzed by ELISA test kits in each plasma (obtained from whole blood after collecting in 3.2% Sodium Citrate in 9:1 ratio) sample of both types of anti-inflammatory activities. These tests were performed by the Rat ELISA kit as per directions provided by manufacturer Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA.

3. Results

3.1 Acute (carrageenan induced rat paw oedema) anti-inflammatory model

The results of anti-inflammatory experiment are shown in Table 1 and Table 2. Table 1 includes results of oedema volume as measured by volume displacement. The values of inhibition of paw oedema were converted into percent inhibition (% oedema regression) that is shown in Table 2. The influence of carrageenan on induction of paw oedema could be clearly visualized from the results of Group I (experimental control) which showed progressive increase in paw volume from 1.177±0.0026 ml to 2.102±0.07 ml through 0 to 4 hours of injection, followed by a decline in next 2 hours to 1.905±0.10 and 1.812±0.16 ml, respectively. The results of Group II (negative control) were also more or less similar, showing progressive increase in paw volume from 1.175±0.0020 ml to 2.096±0.14 ml up to first 4 hours followed by slight decrease during 5th and 6th hours. The decline in paw oedema without any drug appears to be due to

natural healing process of the body (Table 1, Table 2).

Rats of group-III (positive control group) were administered with standard anti-inflammatory drug indomethecin, under the influence of which injection of carrageenan could not cause paw oedema as significant as in group-I and group-II animals. Oedema volume increased from 1.169±0.0016 ml (BCPV) to 1.485±0.08 ml at 2nd hour followed by a declining trend of paw oedema to the status of normal paw volume (BCPV) during 6 hour regimen at 6th hour (Table 1). Thus as compared to control, indomethecin treated animals showed significant inhibition of paw oedema from 1st hour (44.89%) reaching to 100% regression in 6 hour duration (Table 1, Table 2). Treatment with different doses of earthworm extract revealed statistically significant dose dependent inhibition of rat paw oedema when compared to experimental control. In Group-IV animals, treatment with 150 mg extract was failed to bring paw volume to normal level. Oedema volume increased from 1.167±0.0016 ml (BCPV) to 1.517±0.08 ml at 3rd hour and then decreased to 1.272±0.07 ml at 6th hour. In terms of percent inhibition, the values were 40.81%, 44.26%, 50.7%, 68.81%, 71.23% and 82.81% for 1 to 6th hour respectively. Treatment of 200 mg EW extract brought paw volume to near normal level in Group-V animals. Oedema volume was increased from 1.176±0.0014 ml (BCPV) to 1.516±0.06 ml at 3rd hour and then decreased to 1.234±0.03 ml at 6th hour. This was equivalent per cent inhibition ranging from 44.9%, to 92.19% for 1 to 6th hour respectively. Results similar to Indomethecin treatment were shown by treatment with 250 mg of earthworm extract. The volume of paw oedema increased from 1.179±0.0011 (BCPV) ml to 1.488±0.04 ml at 2nd hour and then decreased to normal paw volume during 6 hour regimen. The values of percent inhibition of oedema during 1st to 6th hour periods were 46.93%, 49.18%, 70.42%, 89.24%, 94.52% and 100% respectively.

Table 1: Anti-inflammatory effect of earthworm extract on carrageenan induced rat paw oedema model

Groups		Mean paw volume increase (ml) at different time intervals						
	BCPV	"0" Hour	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour	6 Hour
Group- I Experimental Control	1.177±0.0026	1.275±0.0022	1.664±0.03	1.784±0.05	1.885±0.04	2.102±0.07	1.905±0.10	1.812±0.16
Group-II Negative Control	1.175±0.0020	1.274±0.0038	1.653±0.06^{ns}	1.776±0.07^{ns}	1.897±0.11^{ns}	2.096±0.14^{ns}	1.879±0.09^{ns}	1.804±0.13^{ns}
Group-III Positive Control	1.169±0.0016	1.268±0.0015	1.438±0.02*	1.485±0.08*	1.389±0.06**	1.285±0.05**	1.215±0.06**	1.168±0.02**
Group-IV EW extract (150 mg)	1.167±0.0016	1.266±0.0012	1.460±0.04*	1.511±0.010*	1.517±0.08*	1.459±0.05**	1.389±0.08**	1.272±0.07*
Group-V EW extract (200 mg)	1.176±0.0014	1.275±0.0019	1.445±0.05*	1.497±0.06*	1.516±0.06*	1.457±0.08**	1.352±0.05**	1.234±0.03**
Group-VI EW extract (250 mg)	1.179±0.0011	1.278±0.0017	1.438±0.05*	1.488±0.04*	1.391±0.05**	1.286±0.06**	1.226±0.02**	1.179±0.03**

Values are expressed as mean ± SE of six animals in each group (n=6). P<0.005

(*) = Significant value when compared to control group.

(**) = Highly significant value when compared to control group.

(ns) = No significant value when compared to control group.

Table 2: Results of difference between treated groups and their basal volume in carrageenan induced paw oedema model for assessing anti-inflammatory activity of earthworm extract

Groups	Difference of mean paw volume of treated groups with their respective basal volume (ml) and % inhibition of inflammation at different time intervals					
	At 1st Hour	At 2nd Hour	At 3rd Hour	At 4th Hour	At 5th Hour	At 6th Hour
Group-I Experimental Control	0.49±0.03	0.61±0.04	0.71±0.06	0.93±0.07	0.73±0.10	0.64±0.16
Group-II Negative control	0.48±0.02^{ns} (2.04 %)	0.60±0.03^{ns} (1.64 %)	0.72±0.05^{ns} (-1.41 %)	0.92±0.06^{ns} (1.08 %)	0.70±0.09^{ns} (4.11 %)	0.63±0.13^{ns} (1.56 %)
Group-III Positive control	0.27±0.02* (44.89 %)	0.31±0.08* (49.18 %)	0.22±0.06** (69.01 %)	0.12±0.05** (87.09 %)	0.05±0.06** (93.15 %)	0.00±0.00 (100 %)
Group-IV EW extract (150mg)	0.29±0.04* (40.81 %)	0.34±0.10* (44.26 %)	0.35±0.08* (50.7 %)	0.29±0.05** (68.81 %)	0.21±0.07** (71.23 %)	0.11±0.07* (82.81 %)
Group-V EW extract (200mg)	0.27±0.05* (44.9 %)	0.32±0.06* (47.54 %)	0.34±0.06* (52.11 %)	0.28±0.08** (69.89 %)	0.18±0.05** (75.34 %)	0.05±0.03** (92.19 %)
Group-VI EW extract (250mg)	0.26±0.05* (46.93 %)	0.31±0.04* (49.18 %)	0.21±0.05** (70.42 %)	0.10±0.06** (89.24 %)	0.04±0.02** (94.52 %)	0.00±0.00 (100 %)

Values are expressed as mean ± SE of six animals in each group (n=6). P<0.005

(*) = Significant value when compared to control group.

(**) = Highly significant value when compared to control group.

(ns) = No significant value when compared to control group.

3.2 Chronic (cotton pellet induced granuloma pouch) anti-inflammatory model

The results of chronic anti-inflammatory experiment are expressed in Table 3. The weight of excised cotton pad (now termed as granuloms) was higher in all 6 groups than the initial weight (20 mg) of the cotton pad. The weight of dry cotton pellet granuloma was maximum 105±1.83 and 103±4.51 mg in experimental control (Group-I) and negative control (Group-II) groups respectively, indicating maximum inflammation in the absence of any anti-inflammatory treatment. Weight (31.3±3.1 mg) of cotton pellet granuloma in indomethecin treated (Group-III) rats was only marginally higher than the initial weight (20 mg) of implanted cotton pellet. But it was remarkably lower when compared to experimental control and negative control groups (Group-I and II respectively) and it showed maximum 70.19% degree

of inhibition of granuloma formation. The influence of treatment of earthworm extracts exhibited clear-cut statistically significant reduction in granuloma formation in dose dependent manner. The weight of dry cotton pellet granuloma for 150 mg, 200 mg and 250 mg earthworm extract treated groups were 62.5±5.49, 54.5±2.72 and 51±4.12 mg respectively. In terms of per cent inhibition of granuloma formation, the values were 40.47%, 48.09% and 51.43% respectively. Thus, it can be concluded that oral administration of earthworm extract has significant anti-inflammatory activity, but the effect was not great enough than that of the standard drug indomethecin.

Table 3: Anti-inflammatory effect of earthworm extract on cotton pellet induced granuloma pouch model

Groups	Weight of dry cotton pellet granuloma (mg)	% inhibition of granuloma formation
Groups-I Experimental Control	105±1.83	Nil
Group-II Negative control	103±4.51^{ns}	1.9
Group-III Positive control	31.3±3.1**	70.19
Group-IV EW extract (150 mg)	62.5±5.49**	40.47
Group-V EW extract (200 mg)	54.5±2.72**	48.09
Group-VI EW extract (250 mg)	51±4.12**	51.43

Values are expressed as mean ± SE of six animals in each group (n=6). P<0.005
(*) = Significant value when compared to control group.
(**) = Highly significant value when compared to control group.
(ns) = No significant value when compared to control group.

3.3 Oxidative stress markers in blood samples

The anti-inflammatory influence of EW extract in albino wistar rats was demonstrated by two methods, carrageenan induced paw oedema and cotton pellet induced granuloma pouch. In order to provide supporting biochemical evidence, status of oxidative stress markers *viz.* TBARS, GSH, SOD and Catalase were also investigated in the blood samples of these experimental rats.

3.3.1 Oxidative stress markers in acute model (carrageenan induced rat paw oedema) of anti-inflammatory activity

The results of oxidative stress markers in carrageenan induced experimental rats are presented in Table 4. In experimental control (Group-I) and negative control (Group-II) groups, it was observed that the level of TBARS was high, while the levels of GSH, SOD and Catalase showed a decrease indicating inflamed condition. In inflamed rats pretreated with standard anti-inflammatory drug Indomethecin (Group-III) and different doses of earthworm extract (Group-IV to Group-VI) lower levels of TBARS and increasing values of both enzymatic (GSH) and nonenzymatic (SOD and Catalase) anti-oxidants was noticed (Table 4). The values of different oxidative stress markers were statistically significant when compared to experimental control. The influence of higher dose (250 mg/Kg body weight) was found to exhibit lesser effect than at lower dose levels (150 and 200 mg/Kg body weight).

Table 4: Effect of earthworm extract on oxidative stress markers in carrageenan inflamed rats

Groups	Treatments	Oxidative Stress Markers			
		TBARS[a]	GSH[b]	SOD[c]	Catalase[d]
Groups-I Experimental Control	Nil	333.5±0.43	1.92±0.09	0.25±0.03	1.43±0.03
Group-II Negative Control	Normal Saline	332.4±0.72^{ns}	1.98±0.11^{ns}	0.27±0.06^{ns}	1.41±0.07^{ns}
Group-III Positive Control	Indomethecin	191.5±0.69**	2.44±0.06**	0.52±0.02*	1.65±0.02*
Group-IV EW Extract	150 mg	189.3±0.55**	2.35±0.09*	0.56±0.07*	1.67±0.07*
Group-V EW Extract	200 mg	229.3±0.31**	2.58±0.05**	0.59±0.04**	1.74±0.05*
Group-VI EW Extract	250 mg	316.7±0.90**	2.34±0.03*	0.061±0.05**	1.85±0.05**

Values are expressed as mean ± SE of six animals in each group (n=6). P<0.005
(*) = Significant value when compared to control group.
(**) = Highly significant value when compared to control group.
(ns) = No significant value when compared to control group.
(a) = Expressed as n moles of MDA/ ml of blood
(b) = Expressed as mg/ml
(c) = Expressed as Units / min / mg protein
(d) = Expressed as µM/ min/mg of protein

3.3.2 Oxidative stress markers in chronic model (cotton pellet induced granuloma pouch) of anti-inflammatory activity

In Table 5 results of oxidative stress markers of cotton pellet induced inflammation in experimental rats are presented. Implantation of carageenan impregnated cotton pellet induces formation of granuloma pouch and pre-treatment with standard anti-inflammatory drug Indomethecin and different doses of earthworm extract causes regression of weight of granuloma pouch, indicating their anti-inflammatory activity. Increased level of TBARS and decreased levels of GSH, SOD and Catalase in experimental control (Group-I) and negative control (Group-II) rats confirm that cotton pallet implantation induces inflammation. Further, declining level of TBARS and

increased levels of GSH, SOD and Catalase in inflamed rats pre-treated with Indomethecin (Group-III) and different doses of earthworm extract (Group-IV to VI) confirm the anti-inflammatory nature of the treatments. Values of different oxidative stress markers were statistically significant when compared to experimental control. EW extract at the dose of 250 mg/Kg body weight was found to exhibit increased level of TBARS then comparison to other treatment groups but lower than the experimental control.

Table 5: Effect of earthworm extract on oxidative stress markers in cotton pellet inflamed rats

Groups	Treatments	Oxidative Stress Markers			
		TBARS[a]	GSH[b]	SOD[c]	Catalase[d]
Groups-I Nil Experimental Control		513±0.82	2.63±0.04	1.61±0.03	1.43±0.03
Group-II Negative Control	Normal Saline	515±1.37^{ns}	2.62±0.02 ^{ns}	1.58±0.04^{ns}	1.45±0.01^{ns}
Group-III Positive Control	Indomethecin	317±0.58**	3.2±0.05 **	2.16±0.06**	1.91±0.06*
Group-IV EW extract	150 mg	319.5±0.47**	2.96±0.13*	1.87±0.02*	1.96±0.17*
Group-V EW extract	200 mg	385±0.36**	2.99±0.03*	2.08±0.08**	1.98±0.04**
Group-VI EW extract	250 mg	412.6±0.48**	3.1±0.11**	2.14±0.07**	2.1±0.11**

Values are expressed as mean ± SE of six animals in each group (n=6). P<0.005
(*) = Significant value when compared to control group.
(**) = Highly significant value when compared to control group.
(ns) = No significant value when compared to control group.
(a) = Expressed as n moles of MDA/ ml of blood
(b) = Expressed as mg/ml
(c) = Expressed as Units / min / mg protein
(d) = Expressed as µM/ min/mg protein

3.4. Cytokines in anti-inflammatory activities

Effects of EW extract on two cytokines TNF-α and IL-10 was analyzed in plasma samples of wistar rats of both (acute and chronic model) anti-inflammatory models.

3.4.1 Cytokines in acute model (carrageenan induced rat paw oedema) of anti-inflammatory activity

The levels of cytokines (TNF-α and IL-10) are often used as marker of inflammatory and anti-inflammatory conditions of the animal; elevated level of TNF – α and declining concentration of IL-10 indicate inflamed situation, while reverse (lowering trend of TNF – α and increasing trend of IL-10) is true of anti-inflammatory effect. In the present study, the influence of EW extract treatment on the level of these parameters in the plasma of carrageenan induced (inflamed) rats (paw oedema model), was determined. The results are shown in table 6. The maximum level of TNF - α was noticed in the plasma of experimental and negative control groups, *i.e.* 114.00±0.57 and 115.83±0.79 pg/ml, respectively and lowest concentration of IL-10 was also exhibited in these control groups *i.e.* 22.67±0.72 and 21.50±0.57 pg/ml respectively. Administration of anti-inflammatory agent indomethacin markedly lowered the level of TNF- α in positive control group (48.17±0.60 pg/ml) and the level of IL-10 was found to be higher (70.00±0.26 pg/ml) as compared to other treatment groups. These values were statistically significant when compared to experimental control group. Different doses of EW extract significantly reduced the concentration of TNF- α and increased the level of IL-10 when compared to experimental control. Concentration of TNF- α in the plasma of 150, 200 and 250 mg EW extract treated groups were respectively 64.66±0.49, 56.00±0.82 and 50.50±0.43 pg/ml and the status of IL-10 in these groups were respectively 48.83±0.60, 56.00±0.37 and 67.17±0.48 pg/ml. Among these EW extract treated groups, only 250 mg treated group showed almost same effect as with indomethacin treated positive control group showed.

Table 6: Anti-inflammatory effect of EW extract on different cytokines in carrageenan inflamed rat (paw oedema model)

Groups	Cytokines	
	TNF-α (pg/ml)	IL-10 (pg/ml)
Experimental Control	114.00±0.57	22.67±0.72
Negative Control	115.83±0.79^{ns}	21.50±0.57^{ns}
Positive Control	48.17±0.60**	70.00±0.26**
EW extract (150 mg)	64.66±0.49**	48.83±0.60**
EW extract (200 mg)	56.00±0.82**	56.00±0.37**
EW extract (250 mg)	50.50±0.43**	67.17±0.48**

Values are expressed as mean ± SE of six animals in each group (n=6). P<0.005
(*) = Significant value when compared to control group.
(**) = Highly significant value when compared to control group.
(ns) = No significant value when compared to control group.

3.4.2 Cytokines in chronic model (cotton pellet induced granuloma pouch) of anti-inflammatory activity

The influence of EW extract treatment on the level of TNF- α and IL-10, in the plasma of other model of inflamed rats (cotton pellet induced granuloma pouch model) was also evaluated. A similar trend of results was observed in this experiment also (Table 7). Plasma level of TNF- α was found to be highest in experimental and negative control groups, *i.e.*, 191.50±0.77 and 189.00±0.58 pg/ml, respectively and lowest concentration of IL-10 was also exhibited in these control groups *i.e.* 15.00±0.37 and 16.50±0.43 pg/ml. Administration of indomethacin (positive control) markedly corrected the level of TNF- α (83.00±1.16 pg/ml) and the level of IL-10 (105.00±0.25 pg/ml). These values were statistically significant when compared to experimental control group. Different doses of EW extract significantly lowered the concentration of TNF- α and increased the level of IL-10 when compared to experimental control. Concentration of TNF- α in the plasma of 150, 200 and 250 mg EW extract treated groups were respectively 107.00±0.73, 93.50±0.67 and 90.00±0.86 pg/ml and the status of IL-10 in these groups were

respectively 57.00±0.46, 69.00±0.58 and 87.80±0.55 pg/ml. Among these EW extract treated groups, only 250 mg treated group reached to nearly result of indomethacin treated positive control group.

Table 7: Effect of EW extract on different cytokines in chronic model (cotton pellet induced granuloma pouch model) of anti-inflammatory activity

Groups	Treatments	Cytokines	
		TNF-α (pg/ml)	IL-10 (pg/ml)
Group-I Experimental Control	Nil	191.50±0.77	15.00±0.37
Group-II Negative Control	Normal Saline	189.00±0.58^{ns}	16.50±0.43^{ns}
Group-III Positive Control	Indomethacin	83.00±1.16**	105.00±0.25**
Group-IV EW extract	150 mg	107.00±0.73**	57.00±0.46**
Group-V EW extract	200 mg	93.50±0.67**	69.00±0.58**
Group-VI EW extract	250 mg	90.00±0.86**	87.80±0.55**

Values are expressed as mean ± SE of six animals in each group (n=6). P<0.005
(*) = Significant value when compared to control group.
(**) = Highly significant value when compared to control group.
(ns) = No significant value when compared to control group.

4. Discussion

Carrageenan induced rat paw oedema model is frequently used as a model of inflammation to determine anti-inflammatory effect of the desired substances. It has also been proved as a useful model in assessing the contribution of mediators involved in vascular changes associated with acute inflammation. Subcutaneous injection of carrageenan into the rat paw produces inflammation resulting from plasma extravasation (Szolcsanyi *et al.,* 1998) [38], increased tissue water and plasma protein exudation along with neutrophil extravasation. These responses occur due to the metabolism of arachidonic acid via the cyclooxygenase and lipoxygenase enzyme pathways (Gamache *et al.,* 1986) [12]. Oedema formation by carrageenan is a biphasic response. The first phase begins immediately after injection of carrageenan and diminishes in 2 hours and is attributed to the release of histamine, serotonin and bradykinin. The second phase begins at the end of first phase and remains through 3 to 6 hours. The second phase of oedema is correlated with elevated production of prostaglandins, oxygen derive free radicals and production of inducible cyclooxygenase (Panthong *et al.,* 2004) [24]. In acute inflammatory response pro-inflammatory agents cannot be eradicated completely that's why chronic inflammation develops which includes a proliferation of fibroblasts and the infiltration of neutrophils and exudation (Arrigoni-Maratellie, 1988; Dunne, 1990) [10]. These cells form granuloma, which can be calculated.

The second experiment of the study involved subcutaneous implantation of compressed cotton pellet in rat for induction of granuloma. This model is widely used to evaluate the transudative and proliferative components of the chronic inflammation. The inflammation consists of 3 phases, which are (1) a transudative phase, defined as the increase in the wet weight of the pellet that occurs during the first 3 hours (2) an exudative phase, defined as plasma leaking from the bloodstream around the granuloma that occurs between 3 and 72 hours after the implantation of pellet and (3) a proliferative phase, measured as the increase in the dry weight of the granuloma that occurs between 3 and 6 days after the implantation (Swingle and Shideman, 1972) [37].

Anti-inflammatory drugs like NSAIDs (non-steroidal anti-inflammatory drugs) decrease the size of granuloma which results from cellular reaction by suppressing granulocyte infiltration, forbidding generation of collagen fibers and inhibiting mucopolysaccharides (Della Loggia *et al.,* 1968; Alcaraz and Jimenez, 1988) [1]. Steroidal anti-inflammatory agents strongly inhibit both transudative and proliferative phases whereas NSAIDs exert only slight inhibition (Swingle and Shideman, 1972) [37]. After 6 to 8 days many cells and undifferentiated connective tissue can be observed beside the fluid infiltration which in turn can be measured by weighing the dried pellets after removal. More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenan (Goldstein *et al.,* 1976) [13]. Results of the present study revealed that treatment of rats with earthworm caused anti-inflammatory response in both groups of rats (carrageenan induced rat paw oedema and cotton pellet induced granuloma pouch) in dose (150, 200, 250 mg/kg) dependent manner and the findings were comparable with the results of indomethacin (standard anti-inflammatory agent) treated rats. The level of carrageenan induced paw oedema was significantly lower than those of the control groups. Treatment of rats with 150 mg EW extract provided lower level of anti-inflammation as paw oedema could not decline to normal during 6 hour regimen. Oedema volume increased from basal volume (1.167±0.0016 ml) to 1.517±0.08 ml at 3rd hour and then decreased to 1.272±0.07 ml at 6th hour. As compared to control values, experimental values (referred as per cent inhibition of oedema) were 40.81%, 44.26%, 50.7%, 68.81%, 71.23% and 82.81% lower for 1 to 6th hour respectively. Treatment of rats with 200 mg EW extract brought paw volume to near normal level in animals. Here again oedema volume first showed an increase from 1.176±0.0014 ml to 1.516±0.06 ml at 3rd hour followed by a decline to 1.234±0.03 ml at 6th hour. Per cent inhibition values were 44.9%, 47.54%, 52.11%, 69.89%, 75.34% and 92.19% for 1 to 6th hour respectively.

The best results were shown by rats treated with 250 mg EW extract and indomethecin because paw oedema disappeared completely during experimental time of 6 hours as indicated by values of normal paw volume. From these findings, it can be assumed that the inhibitory effect of the extract of earthworm *E. eugeniae* on carrageenan-induced inflammation could be due to the inhibition of the enzyme cyclooxygenase,

leading to the inhibition of prostaglandin synthesis.

The rats treated different doses of EW extract before implantation of the cotton pellet (chronic test) also exhibited statistically significant reduction in granuloma formation in dose dependent manner. Weight of dry cotton pellet granuloma for 150 mg, 200 mg and 250 mg EW extract treated groups were 62.5±5.49, 54.5±2.72 and 51±4.12 mg and their per cent inhibition of granuloma formation were 40.47%, 48.09% and 51.43% respectively. However the anti-inflammatory activity of earthworm extract was much lower than that of the standard drug indomethecin which showed granulomar weight of 31.3±3.1 mg and per cent inhibition of 70.19%. The present results dealing with anti-inflammatory activity are in accordance with results of a series of experiments conducted on the influence of petroleum ether extract of earthworm *Lampito mauritii* on anti-inflammatory activity which was maximum at 160 mg/Kg dose (Yegnanarayan *et al.*, 1987, 1988; Ismail *et al.*, 1992) [42, 15]. Balamurugan *et al.* (2009) [4] noticed that administration of earthworm extract of *Lampito mauritii* in various concentrations (50-200 mg/Kg) reduced the inflammation in histamine induced rat paw oedema and turpentine oil induced granuloma pouch models.

These results suggested that earthworm extract exerts its anti-inflammatory effect by decreasing granuloma formation because it is assumed that it decrease the number of fibroblasts and demote synthesis of collagen and mucopolysaccharides and in this way it can suppress the proliferative phase of granuloma formation.

The influence of earthworm extract on different biochemical markers of antioxidant activity had also been studied in the blood samples of male Wistar rats through acute and chronic anti-inflammatory models. These parameters included reduced glutathion (GSH), superoxide dismutase (SOD), thiobarbituric acid reactive substance (TBARS) and catalase (CAT). In both the experiments, it was observed that the values of TBARS were significantly lower as compared to the control rats while the levels of other three parameters were significantly higher. More or less similar results were noticed in rats treated with standard anti-inflammatory agent indomethecin. Therefore, it may be concluded that inhibition of inflammation can also be considered to a part of antioxidant activity.

Findings of present study are in the agreement of Balamurugan *et al.* (2007) [3]. They have reported anti-inflammatory potential of *Lampito mauritii* in carageenan induced rat paw oedema and turpentine oil induced granuloma pouch model and revealed that the oral administration of the earthworm extract in rats demonstrated a significant increase in the content of antioxidant enzymes SOD, CAT and GSH which diminished by paracetamol treatment. TBARS concentration was found to be significantly lowered after treatment with earthworm extract, confirming its anti-inflammatory capacity.

Tumor necrosis factor (TNF-α) is a cytokine involved in systemic inflammation which mediate different functions like stimulation of acute phase reaction, fever induction, death of apoptotic cells, inflammation, inhibition of tumorigenesis and viral replication etc. TNF is produced by a variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts and neurons. Large amounts of TNF are released in response to causative agents of inflammatory agents such as lipopolysaccharide, carrageenan, bacterial products and Interleukin-1 etc. IL-10 is one of the cytokines concentration of which is increased during suppression of inflammation because it down regulates the inflammatory reactions. Studies have shown that IL-10 acts as a potent macrophage deactivator, which blocks TNF-α and other pro-inflammatory cytokines (Trushin *et al.*, 2003) [39]. Primarily, it is secreted by activated monocytes, macrophages and Th1 and Th2 cells (Rao, 2005; Mak and Saunders, 2006) [31, 20]. In the present study it was found that treatment of rats with earthworm extract could down-regulate TNF-α and up-regulate IL-10 concentration in both the inflammatory models. Further evidence of dose dependant inhibition of inflammation, as observed by decreasing volume of paw oedema and weight of cotton pellet granuloma, was provided by the determination of values of TNF-α and IL-10. No previous research work has been done to demonstrate the effect of earthworm extract on anti-inflammatory activity in terms of the measurement of TNF-α and IL-10.

From the results of both anti-inflammatory activities including status of anti-oxidant markers and cytokines, it may be concluded that EW extract have significant anti-inflammatory activity because it showed almost similar results as with standard drug indomethacin.

5. Conclusion

The results of both anti-inflammatory and antioxidant activity, revealed that treatment of rats with oral administration of earthworm extract provide considerable anti-inflammatory and antioxidative protection.

6. Acknowledgement

Authors are highly thankful to Defense Research and Development Establishment (DRDE), Gwalior, M.P., India for providing financial assistance.

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