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Preliminary evaluation of the larvicidal efficacy of coelomic fluid of *Eudrilus eugeniae* on anopheles mosquito

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ABSTRACT:*The coelomic fluid (CF) of the earthworm Eudrilus eugeniae exhibits a wide variety of biological activities. A study was made to monitor the larvicidal effect of the coelomic fluid on the larvae of Anopheles mosquito. Bioassay was made using different concentrations to find out the median lethal concentration (LC_{50}). The LC_{50} value obtained was 25% (400µl/ml) against the fourth instar larvae whereas LC_{90} value was 45% (400µl/ml). Protein and carbohydrate were quantified in the larvae that were subjected to the CF-treatment under varying concentrations. SDS PAGE revealed new protein bands in the larvae treated with 30% concentration suggesting the probable expression of stress proteins. Protease activity was assessed in the larvae treated with different concentrations of CF. There is significant increase in the protease activity with the increase in the treatment concentration with CF suggesting significant role of the protein under stress. The finding of this study clearly demonstrated the potent larvicidal property of the CF which is evident from its ability to kill the developmental stages of the Anopheles mosquito vector species.*

KEYWORDS: *Coelomic Fluid, Eudrilus euginiae, larvicidal activity, stress protein, Anopheles mosquito*

I. INTRODUCTION

The coelomic fluid (CF) of the earthworm has agglutinating [1], cytotoxic [2] , proteolytic[3,4], hemolytic [5,6,7,8], muscle contractive [9], antibacterial [7,10], β-1,3-glucan- and lipopolysaccharide-binding and mitogenic [11] [8] activities. In view of this, the present study was designed to survey the toxicity of coelomic fluid to the larvae of mosquito as a search for bioactive component to have potential larvicidal property. The CF of earthworm contains cells and many molecular components involved in innate immunity. Among those are glycoproteins of lectin character, contribute to recognition of foreign material in the coelomic cavity by binding to carbohydrates. Moreover they cause its immobilization by agglutination and destruction by membrane lysis. Malaria is a parasitic disease from which more than 300 million people suffer yearly throughout the world. To combat this disease, many phytochemicals derived from plant resources have been investigated to act as larvicides, insect growth regulators, repellent and ovipositional attractants having deterrent activities observed by many researches [12].

Mosquitoes in the larval stages are attractive targets for pesticides because they breed in water and thus, are easy to deal with them in this habitat. It is known that larvicides play a vital role in controlling mosquitoes in their breeding sites. Although various biocontrol measures are in vogue, their effective control of larval mosquitoes has been highlighted [13, 14]. By using synthetic chemicals with insecticidal properties such as organochlorines, organophosphates, carbamates and pyrethroids have proven to be the most important effective method to control mosquitoes and other insect pests all over the world. Nevertheless, their extensive and indiscriminate applications fostered not only environmental and health concerns but also widespread development of resistance by mosquitoes and unwanted toxic or lethal effects on non-target organisms. Due to such undesirable features of chemical insecticides, several investigators have resorted to explore plant resources to find alternate and ecofriendly compounds with potent anti-mosquito activity [15, 16].In fact, many researchers have reported on the effectiveness on the plant extracts on essential oils against mosquito larvae [17]. New botanical natural products are effective, environment-friendly, easily biodegradable, inexpensive and readily available in many areas of the world, without any ill effect on non-target organisms and have novel modes of action [18].

CF of earthworm is believed to have many bioactive compounds. As earthworms are abundantly available in India, and known for such bioactive functions, the present study was designed to screen for the larvicidal effect of coelomic fluid of the earthworm *Eudrilus eugeniae* on the larvae of *Anopheles* mosquito [19, 20].

II. MATERIALS AND METHODS

Target Animals : To study the mosquito larvicidal effect of the coelomic fluid of *Eudrilus eugeniae*, *Anopheles* mosquitoes were obtained from the wild and identified based on their morphological characteristics features.

Preparation of coelomic fluid

Earthworm- Eudrilus eugeniae : Worms used in this study was *Eudrilus eugeniae.* The earthworms were collected from the Periyar Research Organization for Bio-technique and Ecosystem (PROBE), Periyar Maniammai University, Vallam, Thanjavur, Tamil Nadu, India.

Collection and preparation of CF : Eearthworms (weighing 15±5Gms) were taken from the culture pit and washed in running distilled water. The worms were then placed on the filter paper to remove excess water droplets and were taken in a nylon mesh rolled to fit inside a glass funnel. The nylon mesh was given connection to the 5 Volt electric shock using an eliminator (step-down transformer). The worms were kept under for 30 minutes shock like this continuously and then the fluid was collected. The collected fluid was centrifuged at 5000rpm for 10 minutes to sediment larger particles and other debris. The supernatant was carefully removed and filter sterilized through 0.2 µm (pore size) syringe filter. The filtrate of the coelomic fluid that was free from any suspended cells / coelomocytes (CF) was stored in aliquots at -20°C for subsequent use [21, 22].

Mosquito rearing : About 100 ml of the ditch water was collected and brought to the laboratory. This water was mixed to the plastic trays containing unchlorinated tap water (1:50) and eggs of the mosquitoes were collected within the college campus by placing this water-filled plastic trays (23×15×6.5cm) with a lining of partially immersed filter paper. The plastic trays were kept at room temperature (26 ± 2°C) with a photoperiod of 16:8 h (L: D) for larval hatching. The larvae were fed with a powdered feed containing a mixture of dog biscuit and baker's yeast (3:1 ratio) [23] [14]. To maintain the mosquito population, mosquitoes were fed on sucrose with cotton, soaked in 10% sucrose solution. A plastic tray (11x10x4 cm) filled with tap water with a lining of partially immersed filter paper was then placed inside each cage to enable the female mosquitoes to lay their eggs. The eggs obtained from the laboratory–reared mosquitoes were allowed to hatch into larvae which were immediately used for toxicity assays.

Larvicidal bioassay : Larvicidal bioassays were performed on late 3rd and early 4th instar larvae of *Anopheles* mosquitoes. Test solution was prepared with coelomic fluid of *Eudrilus eugeniae* at different concentrations ranging from 10%, 20%, 30% and 0% (400µl/ml) by diluting the stock solution with double distilled water and these experiments were performed in triplicates. For mortality studies, 6 larvae each of fourth instar were introduced in plastic tissue culture dishes of 35 mm diameter and 10 mm height. The evaluation of mortality rate was performed by verifying the number of dead larvae at the end of 24hours. The larvae were considered dead when they did not respond to stimulus with a Pasteur pipette.

Dose response bioassay : Coelomic fluid of earthworm prepared was subjected to dose-response bioassay for larvicidal activity. From the collected coelomic fluid, four different concentrations of 10%, 20%, 30% and 40% (400µl/ml) were prepared in sterile distilled water containing larval food (dried-yeast powder; 0.2 mg/ml). Six larvae of *Anopheles* at the fourth instar were introduced into sterile culture dishes (35 mm dia; 5 ml capacity) containing appropriate concentrations mentioned above. The control group consisted of only 4ml of double distilled water with food. The evaluation of mortality rate was performed by verifying the number of dead larvae at the end of 24hours.

Mortality correction and Statistical analysis : The percentage mortality observed (% M) was corrected using Abbott's (1925) formula during the observation of the larvicidal potentiality of the coelomic fluid of *Eudrilus eugeniae.*

Corrected percentage of mortality (% M) : The Corrected percentage of mortality is determined according the following equation:

$$[(A-B)/A] \times 100$$

Where,

A – Number of surviving larvae in the control

B – Number of surviving larvae in the test.

Experiment with more than 25% mortality in control was discarded and repeated. The average larval mortality data were subjected to Probit analysis [24] for calculating LC_{50}, LC_{90} and chi-square values.

Sample preparation for biochemical analysis : The treated and untreated late third / fourth instar larvae were collected from the experimental set up for the protein and carbohydrate analysis. Larvae were anesthetized with ice and were homogenized using a micro-pestle. The homogenates were then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were frozen at -20°C until use.

Biochemical analysis : Exactly, 250 µl of the Anopheles mosquito larval sample (treated and control) was used for estimations. The total protein was extracted using Folin-ciocalteau reagent to develop blue colour; subsequent quantification at 650nm was carried out with Bovine Serum Albumin as standard [25]. For carbohydrate estimation, phenol sulphuric acid method at 490nm was carried out with sucrose as standard [26].

Protein profile of Anopheles mosquito larvae

Protein profile of the Anopheles mosquito larvae subjected to coelomic fluid treatment was studied by SDS-PAGE [27] with suitable modifications in the sample preparation as explained below.

Sample preparation for SDS-PAGE by chloroform methanol wash method : After 24 hours, larvae that survived the treatment with coelomic fluid were collected and homogenized with PBS (pH 7.0) in a 2ml microfuge. The tubes were centrifuged at 10,000 rpm for 10 minutes to deposit the debris and the clear supernatant was transferred into a clean dry sterile microfuge. To 200 µl of treated and untreated samples, 480 µl of methanol, 160 µl of Chloroform and 640µl of distilled water were added and spun at 10,000 rpm for 5 minutes. The top aqueous phase was removed and then added with 300µl of methanol. The mixture was spun at 10,000 rpm for 30 minutes. Supernatant was totally removed and the pellet was air-dried to obtain the protein sample. This protein was loaded by mixing with sample loading buffer and separated on a 15% Acrylamide gel.

Determination of molecular mass : The samples of mosquito larvae were run on SDS-PAGE with concurrent run of standard protein markers. The molecular mass of the protein samples were determined using Quantity One Software™ on BioRad Gel Documentation System.

Determination of protease activity : Proteases are the enzymes that catalyze the splitting of proteins into smaller peptide fractions and amino acids by a process known as proteolysis. Gelatin is the denatured product of collagen, and is an abundant protein. A special agarose gel plate was made for this study. Presence of protease activity will exhibit a zone of digestion as lighter area around the well. And the area of diffusion was visualized by precipitating the undigested proteins.

Sample preparation for protease activity : Sample for the determination of protease activity by Gelatin Diffusion Assay was obtained by processing the mosquito larvae as explained for the protein profile.

Gelatin diffusion assay : Modified Maskel and Di Capua's (1988) method of *invitro* gelatin lysis assay was employed to find the protease activity in the protein samples of the treated and untreated mosquito larvae. Briefly, a solution of 0.5% gelatin and 1.5% agarose were prepared in 0.1M Tris-HCl, pH 7.0 (Tris). Exactly 5ml of gelatin-agarose gel was poured onto a rectangular dish of 8 x 4 cm. Wells of 6 mm were cut into solid agarose and filled with 30µl of coelomic fluid. After 4 hrs of incubation at 37°C, the gelatin-agarose gel was precipitated with 5ml of solution containing 15g of $HgC1_2$ and 20ml 12N HC1 in 80ml distilled water. The diameter of the clear circle around each well was measured [28].

Effects on the growth and development of larvae of Anopheles mosquito

The larvae that were subjected to treatment of coelomic fluid were carefully examined for any abnormalities in the normal course of development.

III. RESULTS AND DISCUSSION

Collection of Coelomic Fluid : Coelomic fluid was collected by electric shock method. During every time of fluid collection, approximately, 1.3ml of fluid was obtained from a group of 40g of worms within 30 minutes.

Effect of CF on mosquito larvae : Mean LC_{50}, LC_{90} and X^2 values of the coelomic fluid of *Eudrilus eugeniae* earthworm against fourth instar larvae of *Anopheles* mosquito were determined and the values were tabulated (Table 1; Fig. 1). Mean LC_{50}value of the coelomic fluid was 25% (400µl/ml) and that of LC_{90} was 45% (400µl/ml). There was no significant difference ($P<0.5$) between the observed LC_{50} and LC_{90} values and that of

the computed (by Probit analysis). This suggested that the coelomic fluid of the earthworm can be a potent larvicide at 25% (400µl/ml). Though in 10% concentration, the coelomic fluid demonstrated 60% and 90% mortality of larvae at the end of 48 and 72 hours respectively; thereafter 100% mortality was observed at the end of 96 hours.

Total carbohydrate in the treated larvae of Anopheles : Total carbohydrate in the control group and treated larvae of *Anopheles* mosquito were determined and tabulated (Table 2). The values were significantly lower by three folds in the treated larvae (30%) in comparison to the control.

Protein determination : Protein was quantified in the treated and control group of larvae for three different concentrations and the values were tabulated (Table 2). When compared to the control, the protein content per gram of larvae gradually increased with the increase in the concentration of the coelomic fluid during the treatment for 24 hours.

Protein profile by SDS-PAGE : SDS-PAGE revealed the presence of proteins of different molecular weights in the treated and untreated samples. There is progressive increase in the protein concentration in the CF-treated larvae from 10 to 30 % (Fig. 2). At 30% of CF, the larvae showed three protein bands of 117.59, 64.98 and 29.38 KDa. The treated and untreated samples appeared to have different kinds of proteins in the present investigation.

Demonstration of protease activity by gelatin diffusion assay : Protease activity was clearly visible from the precipitation of gelatin present in the agarose medium. The digestion of gelatin confirmed the proteolytic activity of treated and untreated samples of *Anopheles* mosquito larvae. Clear zones were observed after 4hrs of incubation of samples at 37^0C. The proteolytic reaction of samples increased rapidly during the first hour of incubation at 37^0C at pH 7. The diameter of zone around each well was clearly viewed by precipitating the undigested gelatin. The study of protease activity of the larvae under different concentrations coelomic fluid of *Eudrilus eugeniae* demonstrated a gradual increase in the diameter of the zone with the increase in the concentration of the treatments (Fig. 2). The diameter of the lysis circle formed by the coelomic fluid–treated larvae at different concentrations of 10%, 20%, 30%and 40% was found to be 10mm, 11mm, 11mm and 12mm respectively. The control group of larvae exhibited a diameter of only 7 mm. The results were tabulated in (Table 3).

Effects on the growth and development : After the treatment, the larvae showed abnormalities in their movement. Some of the larvae were found to be less active and some were found to be inactive. The abnormalities in their movement were dose dependent. The mobility of the larvae was normal in the 10 % CF treatment. In 20 and 30 % treatment with CF, the larvae showed vigorous body movement in the first day itself. Curling up of the larva was observed in the 20 and 30% treatment with CF. However, discoloration of the larvae was not prominent in any of the treatment.

Malaria is probably the most sensitive vector-borne disease [29]. Malaria transmission depends on mosquito vector and their distribution depends on breeding habitat availability, suitability and productivity [30]. Presently, organochlorine, organophosphate and synthetic pyrethroid insecticides are being used for public health sprays and successive changes in the insecticides result in multi-insecticide-resistant malaria vectors [31]. In India, malaria vectors are resistant to organochlorine, organophosphate and pyrethroid insecticides [32] especially; Anopheles sp.that has been noted by World Health Organization (World Health Organization, 1989). Control of mosquito larvae becomes a very pertinent issue in controlling the rapid replication of mosquitoes in the management of vector- borne diseases. In the present study, the CF of *Eudrilus eugeniae* exhibited promising larvicidal activity against *Anopheles* mosquitoes.

Although CF collection is a costly affair than other larvicidal agents, such as temephos, and Malathion, it has the advantage of being effective and ability to prevent the development of pest resistance.

High larval mortality (90%) was recorded in larvae treated with 40% of CF. This may be due to the chemical constituents present in the CF that arrest the metabolic activities of the larvae. CF may have inhibiting influence on neurosecretory cells or may act directly on epidermal cells which are responsible for the production of enzymes for the tanning or cuticular oxidation process [15,31]. This is in agreement with the results of the studies conducted with the undiluted coelomic fluid of a similar earthworm species *Eisenia fetida* that inhibited egg hatching upto 100% in *Meloidogyne javanica* – a root knot nematode [33].Even at lower concentrations of CF (10 and 20%), after prolonged exposure for over 72 hours, larvae became inactive with high degree of

disturbance in behavior of the larvae as curling up, vigorous body movements which are the characteristics of neurotoxicity [31]. This is similar to the study

Several studies have reported that the total protein and carbohydrate contents were reduced along with certain amino acids in the phytochemical extract-treated larvae suggesting that the treatment has lowered feeding, improper utilizations of digested foods and interference with the hormones regulating the protein synthesis leading to reduced nutrient profile [31]. In contrast to this, the total carbohydrate and protein were seen to be elevated significantly in the CF-treated larvae. This elevated protein level was evident from the SDS-PAGE showing strong bands in comparison to the control. CF treatment probably induced stressful condition resulting in the expression of the stress related proteins. Protease activity of the CF-treated-larvae was also elevated in the higher concentrations which correlate with the elevated protein content in the larvae revealed by the biochemical analysis.

IV. CONCLUSION

To conclude, an attempt has been made to evaluate the role of coelomic fluid of the earthworm, *Eudrilus ugeniae* in mosquito larvicidal activity. The results reported here open the possibility for further investigations on the efficacy of larvicidal properties of coelomic fluid of the earthworm. The finding of this study clearly demonstrated the potent larvicidal property of the coelomic fluid evident from its ability to kill the developmental stages of the Anopheles mosquito vector species.Further studies are needed to evaluate the identity of the bioactive components of this fluid and its systemic effects on target mosquitoes, which would eventually enable the application of the coelomic fluid as an eco-friendly biocidal agent for the effective control of the mosquito vectors.

Table: 1: Percentage mortality of larvae of *Anopheles* mosquito under the treatment of CF of *Eudrilus eugeniae*

S. No	Concentrations (µl)	No. of larvae exposed	No. of mortality	% of mortality	LC_{50}(µl)	LC_{90}(µl)	X^2(df)
1	Control	6	1	-			4.16(4)
2	10%	6	1	-			4.16(4)
3	20%	6	3	40%	25%	45%	1.50(4)
4	30%	6	4	60%			0.66(4)
5	40%	6	5	80%			0.16(4)

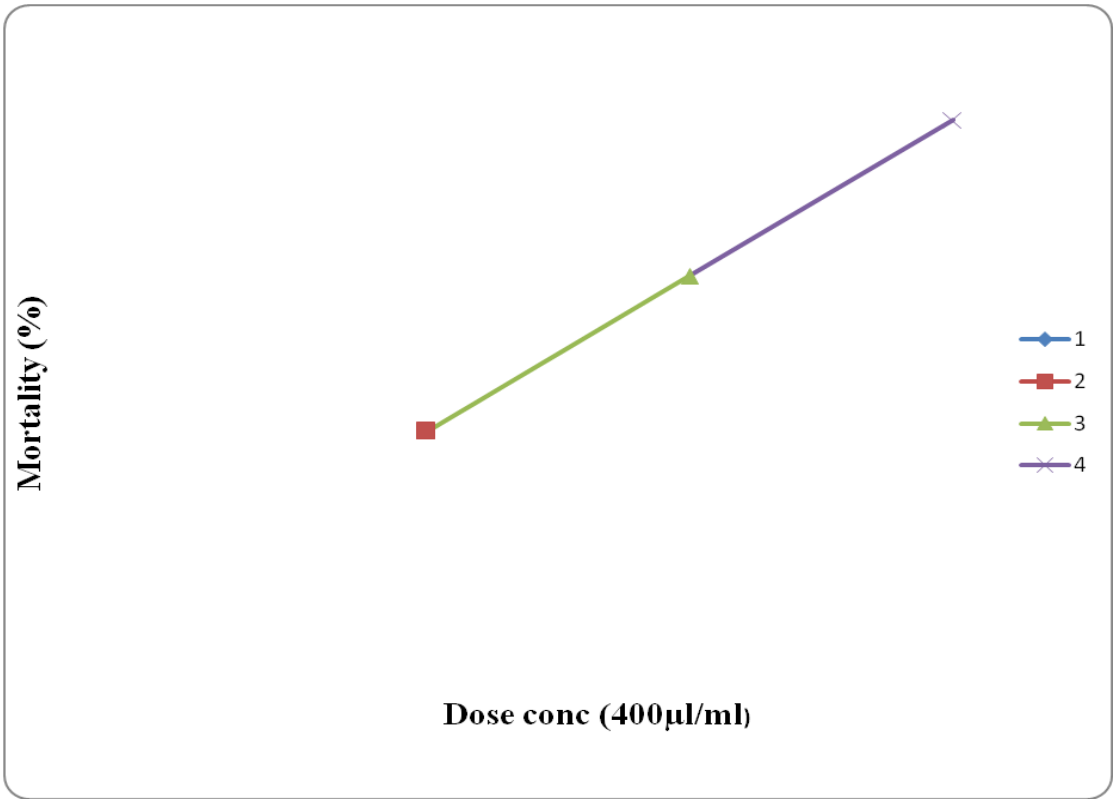
Table: 2- Showing the concentrations of treated and control group of larvae of *Anopheles* mosquito.

S. No	**Concentrations(µg)**	**Total Carbohydrate (per 0.1 g)**	**Protein (per 0.1 g)**
1	10%	12µg	17.4µg
2	20%	7µg	20.0µg
3	30%	6µg	21.2µg
4	40%	4µg	23.8µg
5	Control	18µg	56.25µg

Table: 3: Diameter of the lysis circle formed by different concentrations of coelomic fluid**.**

S. No	Concentrations(µl)	Diameter of the circle
1	control	7mm
2	10%	10mm
3	20%	11mm
4	30%	11mm
5	40%	12mm

Fig 1: Regression line of larval mortality of *Anopheles* treated with different concentrations of CF of *Eudrilus eugeniae* earthworm



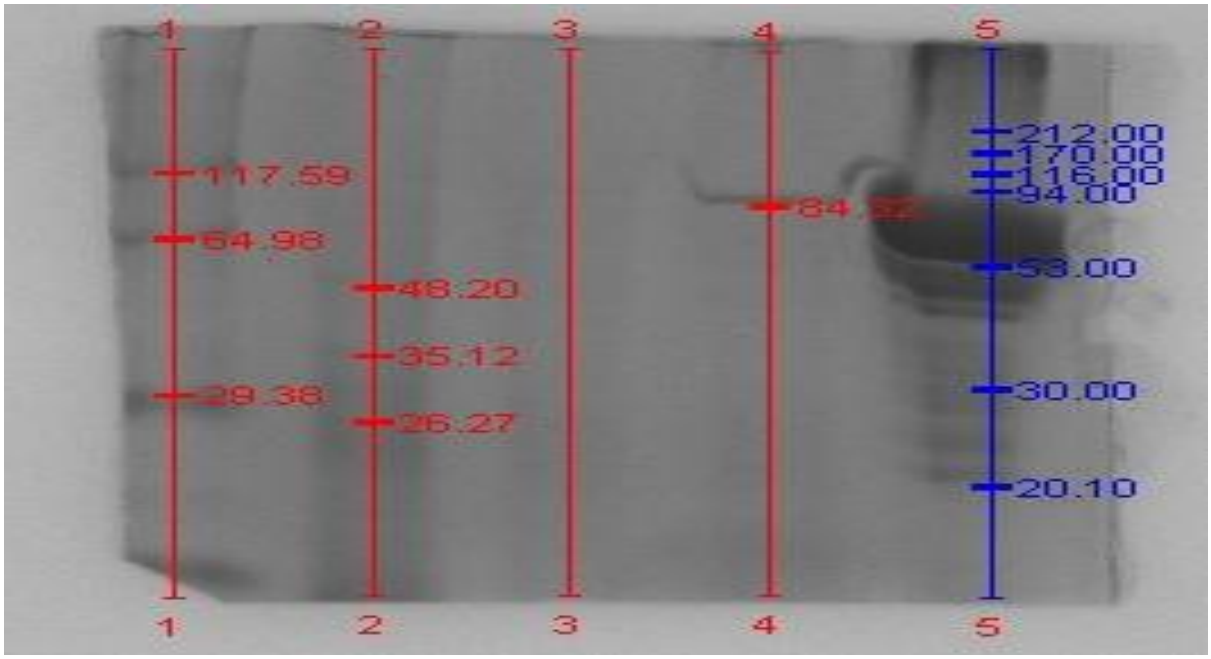


Fig 2: SDS-PAGE analysis (with computed Mol. Wt. of the proteins with reference to the virtual standard analyzed on Bio-Rad Quantity One Software) of the larvae treated with different concentrations of CF. Lane 1- 30%; Lane 2 – 20%; Lane 3- 10%; Lane 4- Control; and Lane 5 – Molecular marker

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