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Evaluation of the Larvicidal Effect of Coelomic Fluid of *Eudrilus eugeniae* on *Aedes* Larvae

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Abstract : *The present study was carried out to evaluate the larvicidal potentiality of the coelomic fluid of Eudrilus eugeniae on the larvae of Aedes mosquito. Mortality was checked at different concentrations of coelomic fluid ie. 10%, 20%, 30% and 40%. Protein and carbohydrate were quantified in the larvae subjected to CF-treatment under varying concentrations. SDS-PAGE revealed new protein bands in the CF-treated larvae which suggests the probable expression of stress proteins. Coelomic fluid assessment by FT-IR Spectroscopy revealed the presence of Arginine-containing protein in the CF responsible for earthworm's immunity and membrane-penetrating property. The findings of the study clearly exhibited the potent larvicidal property of CF, which is evident from its ability to kill the larvae of the Aedes mosquito vector species.*

Keywords *: Coelomic fluid, Aedes, larvicidal potential, Eudrilus eugeniae, SDS-PAGE, FT-IR Spectroscopy, stress proteins, Arginine.*

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Introduction :

Dengue has become a global problem since the second world war and is common in more than 110 countries(Bhatt et al., 2013). Each year between 50 to 530 million people are infected by the disease and approximately 10,000 to 20,000 deaths are caused (Carabali et al.,2015). The rate of dengue has increased 30-fold between 1960 and 2010 (WHO,2009), due to the combined effects of urbanisation, population growth, increased international travel and global warming (Whitehorn and Farrar, 2010). It is caused by the Dengue-virus having five different subtypes called serotypes (Normile,2013) primarily transmitted by *Aedes* mosquitoes. Due to the presence of many beneficial characteristics in them, a number of plant products have been evaluated against vector mosquitoes to explore the possibility of their use in the integrated mosquito control programme.

It has been found that the coelomic fluid of earthworms has 40 different proteins and exhibits complex biological effects, such as, agglutinating (Mohrig et al,1989), cytotoxic (Kauschke and Mohrig,1987), proteolytic (Leipner, 1991 and

Mohrig et al.,1989), haemolytic (Andrews and Kukulinsky,1975 and Roch et al.,1990), muscle contractive (Sekizawa et al.,1997), anticancerous, β-1,3-Glucan and lipopolysaccharide binding and mitogenic (Hanusova et al.,1999), antibacterial (Valembois et al.,1982) activities because of the presence of several bioactive proteins in it. In view of this, the present study was designed to survey the toxicity of coelomic fluid to the larvae of the mosquito, as a search for bioactive components which have a potential larvicidal property.

Coelomocytes located in this fluid are responsible for innate cellular immune functions such as phagocytosis and encapsulation against parasites and pathogens. The effector cells participate mainly in cellular mechanism but chloragocytes and granulocytes of coelomocytes may produce humoral factors which may mediate the cellular and humoral response as well. These properties are responsible for a unique and highly efficient immune system of earthworms. So, larval killings may result from the combined action of the humoral immune factors like agglutinin (viz.lactin), lysosomal enzymes (viz.acid phosphate) and various cytotoxic and proteolytic molecules acting alongside the cellular immune response.

Mosquitoes in the larval stages are attractive targets for pesticides, because they breed in water and thus, are easy to deal with in this habitat using various larvicides. 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 exploring plant resources to find alternate and ecofriendly compounds with potent anti-mosquito activity (Jeyabalan et al.,1999 and Amer and Mehlhorn, 2006). 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.

The present study throws light on the larvicidal effect of coelomic fluid of earthworm *Eudrilus eugeniae* on the larvae of *Aedes.* As earthworms are abundantly available in India, this may prove to be a highly effective, eco-friendly biocidal agent to combat the havoc of dengue, chikungunya etc.

Materials and Methods :

The earthworms used in the study were *Eudrilus eugeniae*. They were obtained from a local organic farmer of Nagma, Naubatpur, Patna, Bihar and cultured under laboratory conditions. Moisture level and temperature were maintained during culture. Adult earthworms were taken from the stock culture.

Collection and preparation of coelomic fluid:- Coelomic fluid was obtained from the earthworms, using the cold shock method. Each time, during fluid collection, approximately 14gms of worms were used which yielded 2.5-3 ml of coelomic fluid within 30 mins.The coelomic fluid that oozed out was collected and centrifuged at 10,000 rpm for 10 minutes to sediment the debris and larger particles. The supernatant was carefully removed using a 100 µl pipette and was stored in eppendorfs at -20°C for subsequent use (Pan et al., 2003 ; Kobayashi et al., 2004).

Mosquito rearing:- Larvae of *Aedes* mosquitoes were collected from the college genetic nursery water tank and identified based on their

morphological characteristic features. These were kept in water-filled plastic trays (36×25×9cm) at room temperature (30°C) and fed a powdered mixture of dog biscuit and baker's yeast in the ratio 3:1. Pupae were transferred to plastic bowls of diameter 12cms. The adults were fed with 10% sucrose solution. A small enamel tray (26×16×4cm) with partially emersed filter paper was filled with tap water and placed inside the net to enable egg-laying after blood meal on Swiss albino mice. Eggs from the laboratory reared mosquitoes hatched into fresh larvae that were used for tests in the experiment.

Dose-response larvicidal bioassay:- Test solutions were prepared at different concentrations, 10%, 20%, 30% and 40% (2ml/5ml) by diluting the stock solution with double distilled water, while the control consisted of only double distilled water. For studies on mortality, 8 larvae each of late 3rd or early 4th instar stage of *Aedes* mosquitoes, were introduced in the plastic tissue culture dishes of 1 cm diameter and 1.5 cm height containing the respective test solutions.Experiments were performed in triplicate. The number of dead larvae at the end of 24 hours was verified when they did not respond to stimulus by a pipette.

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

$$[(A-B)/A]x100$$

where, A is number of surviving larvae in the control, and

B is number of surviving larvae in the test.

Sample preparation for biochemical analysis:- The treated and untreated larvae were collected in separate eppendorfs for the protein and carbohydrate analysis, fit into cryostand and freeze-thaw was performed using liquid nitogen at -196°C. The larvae were then homogenized, using a micro-pestle and PBS(Phosphate buffered saline at pH 7.0) added for dissolution and homogenisation. The prepared sample was stored at -20°C, until further use.

Extraction of total proteins:- The total protein was extracted using TCA-Acetone method for protein extraction (Valerie 2007). The extracted protein pellets were dissolved in 50µl PBS for sample preparation.

Biochemical analysis of extracted protein:- Quantification of the total protein in sample was done using BCA (Bicinchoninic Acid) method for protein quantification at 570nm using Bovine Serum Albumin (BSA) as standard (Lowry et al.,1951).

Biochemical analysis of carbohydrates:- For carbohydrate estimation and quantification, the phenol sulphuric acid method was carried out at 490nm and 595nm, with glucose as standard (Dubois et al.,1956) with suitable modifications.

Protein profile of *Aedes* larvae by SDS-PAGE:- Protein profile of the *Aedes* mosquito larvae, subjected to coelomic fluid treatment and control was studied by SDS-PAGE i.e, Sodium Dodecylsulphate-polyacrylamide Gel Electrophoresis (Laemmle,1970), with suitable modifications on 12% polyacrylamide gel with concurrent run of standard protein markers.

FT-IR Spectroscopy:- FT-IR Spectroscopy was employed to analyse the functional groups present in coelomic fluid of *Eudrilus eugeniae*, that may explain its larvicidal potentiality.

Effects on the motility and activity of *Aedes* larvae subjected to treatment:- The larvae that were subjected to treatment of coelomic fluid were carefully examined during treatment for any abnormalities and movement.

Statistical analysis:- The results are represented as Mean±SE.Student's t-test was also

performed, criterion for statistical significance was set as $P<0.05$.

Results and Discussion :

The results of the present work showed that there was no significant change in weight of earthworms after coelomic fluid extraction. The earthworms survived and were ready for fresh extraction after 3-4 weeks.

Table 1. Percentage mortality of larvae of *Aedes* mosquito under treatment of coelomic fluid of *Eudrilus eugeniae*.

S. No.	Concentration (µl) of coelomic fluid	No. of Larvae Exposed	No. of Dead Larvae	% of Mortality
1.	Control	8	0	00.0%
2.	10%	8	1±0.00	12.5%
3.	20%	8	2±0.33	25%
4.	30%	8	3±0.33	40%
5.	40%	8	5±1.202	62.5%

Values are Mean±SE (n=3).

*Significant at $P<0.05$ as compared to control.

There was no or negligible mortality observed in control group whereas the coelomic fluid showed significant increase in mortality with increase in the concentration. Table 1 shows that the coelomic fluid of earthworms can be a potent larvicide at 30% and 40% concentration. Upto 63% mortality was observed in 40% treated larvae by the end of 48 hrs.

During the treatment, the larvae showed abnormalities in their movement. Mobility was normal in the control and 10% coelomic fluid treatment while the larvae treated with 20%, 30% and 40% showed vigorous body movement in the first few hours of treatment and activity decreased with passing time. Curling up of the larvae was observed in the 20% and 30% treated larvae, mainly in 30%. No discoloration of larvae was seen in any of the treatments.

Table 2. Showing the protein concentration in treated and control groups of larvae

S. No.	Concentration(µl) of coelomic fluid	Total Protein of sample (µg/µl of sample)
1.	Control	8.252±0.219
2.	10%	3.895±0.010*
3.	20%	3.921±0.004*
4.	30%	6.139±0.140*
5.	40%	8.904±0.136

Values are Mean±SE (n=3) .

*Significant at $P<0.05$ as compared to control.

Table 2 shows the protein content to be significantly lower in the treated larvae as compared to the control group ie. less by 53% in the 10% coelomic fluid-treated larvae as compared to control. But interestingly, protein content gradually increased with increase in concentration of coelomic fluid and even exceeded that of control in 40% treated larvae.

Table 3. Showing the carbohydrate content in treated and control groups of larvae

S.No.	Concentration(µl) of coelomic fluid	Total Carbohydrate in sample(µg/µl)
1.	Control	1.390±0.213
2.	10%	1.510±0.253
3.	20%	1.854±0.452
4.	30%	1.875±0.446
5.	40%	2.390±0.710

Values are Mean±SE (n=3).

Not significant at $P<0.05$ as compared to control.

Table 3 shows that the carbohydrate values were found to slightly increase in the treated larvae in comparison with the control group. Carbohydrate was most abundant in 40% coelomic fluid treated larvae ie. 77% increase as compared to control but the changes were not significant as compared to control group.

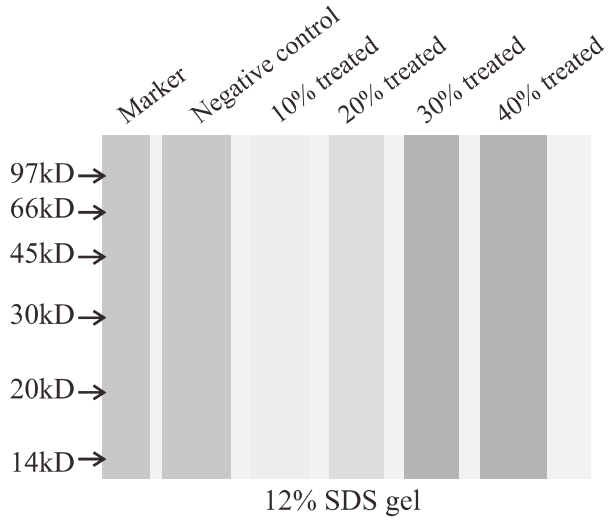

Fig. 1. SDS-PAGE analysis of the larvae treated with different concentrations of coelomic fluid and control, respectively on 12% Polyacrylamide Gel.

SDS-PAGE shown in Fig. 1 revealed the presence of proteins of different molecular weights in the treated and untreated samples.The larvae showed protein bands in varying ranges ie. 15-18kD, 28-30kD, 40-45kD and 70-90kDs.The treated and untreated samples appeared to have the same as well as slightly different kinds of proteins in this investigation.There was a progressive increase in the protein concentration in the coelomic fluid-treated larvae from 10% to 40%.

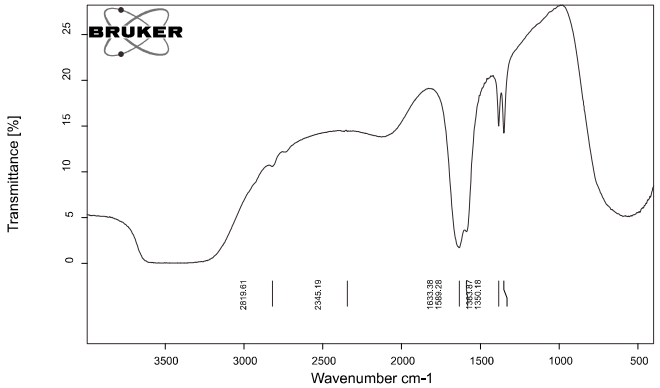

Fig. 2. Showing the spectrum obtained when coelomic fluid of *Eudrilus eugeniae* was assessed by FT-IR Spectroscopy

Spectrum of coelomic fluid showed a broad spectrum band at 3500/cm (Fig. 2). This is mainly due to the -OH group. We also noted 1633/cm of wavenumber, which indicates the presence of amino acid Arginine in the coelomic fluid of *Eudrilus eugeniae*. Bands at 1623/cm and 1663/cm were not seen indicating the absence of lysine and glutamic acid respectively. The band at 1384/cm observes C-H bending vibration and 1350/cm is attributed to Phenol-Hydroxyl stretching. 650/cm band is observed due to plane bending vibration of intermolecular H-bonding.

High larval mortality (upto 90% in 48hrs) was observed in larvae treated with 40% of coelomic fluid (Jaabir et al.,2014). This may be due to the chemical constituents present in the coelomic fluid that arrest the metabolic activities of the larvae. Coelomic fluid may have an 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 (Jeyabalan amd Murugan,1999 and Raghavendra and Subbarao 2002). 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 (Rostami et al.,2013).

Even at lower concentrations of coelomic fluid(10% and 20%), after prolonged exposure for over 72 hours, the larvae became inactive with a high degree of disturbance in the behaviour of the larvae as curling up, vigorous body movements which are the characteristics of neurotoxicity (Raghavendra and Subbarao, 2002). These responses suggest that even a lower dose/concentration of coelomic fluid can mediate stress responses, if exposed for a longer duration of time.

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 (Raghavendra and Subbarao, 2002]. In contrast to this, the total carbohydrate and protein were seen to be elevated significantly in the coelomic fluid-treated larvae. This elevated protein level was evident from the SDS-PAGE showing strong as well as new bands in comparison to the control. Coelomic fluid treatment probably induced stressful conditions in the larvae resulting in the expression of the stress related proteins. The slightly elevated levels of carbohydrate can be explained as probably the expression of agglutinins, which are glycoproteins in nature. The FT-IR Spectroscopy further confirmed the presence of Arginine containing proteins in the coelomic fluid. Arginine is popular for its membrane-penetrating, anti-hypertensive properties responsible for immunity. This protein may be some proteolytic enzyme responsible for the breakdown of larval protein or some other haemolytic or cytotoxic substance which leads to larval death.

Conclusion:

The results reported in present study revealed the potent larvicidal properties of coelomic fluid which has the ability to kill the developmental stages of the *Aedes* mosquito vector species. It opened the possibility for further investigations on the efficacy of coelomic fluid of earthworms as larvicide. Further studies are needed to evaluate the identity of the bioactive components of this fluid and its systemic effects on target mosquitoes. The coelomic fluid will be an eco-friendly biocidal agent for the effective control of the mosquito vectors.

By finding a suitable mode for the application of coelomic, deadly diseases like dengue, chikungunya and zika virus can be checked. Coelomic fluid will prove an efficient biological agent with no ill effects on the ecosystem that the chemical pesticides cause.

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