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ANTIMICROBIAL ACTIVITY OF EARTHWORM EXTRACT, *EUDRILUS EUGENIAE* AGAINST FISH BACTERIAL PATHOGENS

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ABSTRACT

The present study was conducted to examine antimicrobial activity of earthworm extract, *Eudrilus eugeinae* against fish bacterial pathogens. 8 bacterial strains (6 gram negative and 2 gram positive) were isolated and identified from diseased fish. The antimicrobial activity of extract from *Eudrilus eugineae* was determined under *in vivo* study by using antimicrobial well diffusion assay. The earthworm extract showed antibacterial activity against isolated pathogenic bacterial strains. The maximum zone of inhibition (21.00 ± 0.57 mm) was shown against *Aeromonas hydrophila* by earthworm extract. The study establishes that earthworm extract from *Eudrilus eugeinae* has great antibacterial potential and can be used to suppress bacterial infection in fishes.

KEY WORDS
Bacteria
Common carp
Eudrilus eugineae, zone of inhibition
Antimicrobial activity

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INTRODUCTION

Diseases and pollution play an important role in aquaculture. Bacterial disease outbreaks impose a significant constraint on the production of fish and shellfish (Bachere *et al*., 1995; Verschuere *et al*., 2000; Gomez *et al*, 2007). Bacterial pathogens have been reported to cause heavy mortality in both cultured and wild fish species in different parts of the world (Bader *et al*., 2003; Joseph and Clerk, 2002). The use of anti microbial drugs in agriculture and aquaculture has led to the emergence of antibiotic resistant bacteria (Schwarz *et al*., 2001; Akinbowale *et al*., 2006).

Extracting and using biologically active compounds from earthworms has traditionally been practiced by indigenous people throughout the world, more particularly in Asia, including India, Myanmar, China, Korea and Vietnam (Ranganathan, 2006). Earthworms have been known for many centuries as a therapeutic drug source for various diseases in China and other parts of the Far East (Ismail, 2005). Studies on earthworm have shown its antipyretic, antispasmodic, detoxic, diuretic, antihypertensive, antiallergic, anticoagulative, antiasthmatic, spermatocidal, antioxidative, antimicrobial, anticancer, antiulceral and anti-inflammatory activities (Wang *et al*., 2007; John and Packialakshmi, 2007; Shobha and Kale, 2007; Balamurugan *et al*., 2009; Cooper and Balamurugan, 2010; Ansari and Sitaram, 2010; Hrzenjak *et al*., 1998).

Recently earthworm protein and its coelomic fluid were reported to exhibit cytolytic, agglutinating, proteolytic, haemolytic, mitogenic, tumorstatic and antibacterial activities (Edwards and Bohlen, 1996; Popovi *et al*., 2001; Cooper, 2005). Very recently Prakash *et al*. (2007) and Balamurugan *et al*. (2007) have reported that the presence of anti-ulceral and anti-oxidative properties of earthworm paste of *Lampito mauritii*.

A significant number of studies have been carried out by researchers across the globe for assessing and evaluating the antibacterial activity of earthworm powder/ paste/extract. However, almost negligible attempts have been made to study this extra-ordinary property of earthworms in fish health management. In order to scientifically analyze some of the ethnomedical uses of earthworm, the present investigation was undertaken to analyze the antibacterial properties of earthworm extract and its use in fish health management.

MATERIALS AND METHODS

The study was carried out at Fish Biotechnology Laboratory and Aquarium Room, Department of Zoology, Chaudhary Charan Singh Haryana Agricultural University, Hisar. The following materials and methods were used during the research work.

Isolation and identification of bacterial strains from fish

Collection of material

Healthy and infected fish (*Cyprinus carpio*) were collected from fish farms/ponds in Hisar (Haryana) and brought to the Fish Biotechnology Laboratory of the department. The external symptoms of the infected fish were recorded. The specimens were kept under laboratory conditions for isolation and the identification of bacterial strains. The earthworms, *Eudrilus eugineae* were collected from Vermicomposting Unit, Department of Zoology, CCSHAU, Hisar.

Culture of bacteria

The tissues and organs (skin lesions, gills and intestine) were dissected out and homogenized by the homogenizer in the glass tube. The supernatant was spread over the nutrient agar (NA) medium under aseptic conditions. The plates were incubated in B.O.D at 30 ± 2ºC for 18-24 hours. Growth on NA plate was observed after 18-24 hours. Pure colonies of bacteria were obtained by further sub-culturing single colonies on NA plates. (Gerhardt *et al*., 1994)

Identification of bacteria

Isolated pure culture bacteria were subjected to a number of important biochemical tests (primary and secondary tests for identificationdescribed by Krieg and Holt (1984) in "Bergey's Manual of Determinative Bacteriology". The identification of bacteria was done with the help of Computer program, PIBWin (Website: *www.soton.ac.in.uk/tnb/pib.htm*).

***In vivo* pathogenicity test**

Healthy fish (common carp) weighing about 20-25g were procured from the fish farms and brought to the laboratory. The fish were acclimated for one week in the laboratory. The fish were fed a normal recommended commercial diet. *In vivo* pathogenicity test was carried out on healthy fish by method as described by Keskin *et al*. (2004).

Pure culture of the isolated and identified bacteria with known viable count were injected intraperitonially (i.p.) into fish @ 0.5 ml per fish. The control fish were inoculated with normal saline. The experiment was done in triplicates with 10 fish in each tub in each replicate. The symptoms of disease appearance were examined and incubation period of different bacteria in fish and longevity of fish were recorded.

Preparation of earthworm paste (Balamurugan *et al.* 2007)

Earthworms (*Eudrilus eugineae)* were obtained from Vermicomposting Unit of the Department of Zoology. Sexually mature clitellated worms were washed with running tap water and then fed with wet blotting paper for 18-20 hours for gut clearance. The gut cleared worms were again washed with distilled water. The worms were kept in plastic trough covered tightly with polythene cover and exposed to sunlight for three days to kill the earthworms. Mucus and coelomic fluid that oozedout formed a brown coloured paste called earthworm paste. After mastication of whole earthworm obtained with mucus and coelomic fluid, a brown coloured paste was formed.

Well diffusion technique (Gram and Melchiorsen, 1996)

Nutrient agar (NA) was cooled at 45ºC and inoculated with the target organism to a final density of 10^7 cells /mL agar media and was poured into the Petri dishes as described by Gram and Melchiorsen, (1996). The plates were kept in Laminar Air Flow for 30 min. to allow solidification.With the help of cork-borer, three wells were prepared in each solidified agar plate and were sealed with water agar.The earthworm extract (50 ml) was inoculated in wells (r = 0.25 cm) with the help of a micropipette.The plates were then incubated at 32 ± 1ºC in B.O.D. incubator and observations were recorded for zone of inhibition around the wells after 24h.The clear zone of inhibition was measured using a centimeter scale and the observations were recorded.

Statistical analysis

The obtained results were analyzed statistically using completely randomized design (CRD) following Snedecor and Cochran (1989).

RESULTS

Isolation of bacterial strains was done form healthy and diseased *Cyprinus carpio* collected from fish farms/ ponds in Hisar. The diseased fish showed symptoms like hemorrhages on head and lateral side of the body, skin erosions, Fin and tail erosion, ulcers and rottening of gill lamella.Isolated pure cultures of bacteria were subjected to a number of important biochemical tests. Eight bacterial strains were isolated and identified from *Cyprinus carpio* (Table 1).

Longevity of *Cyprinus carpio* inoculated with different pathogenic bacteria

Table 2 shows the longevity in days of fish infected with different

Table 1: Incubation period of different pathogenic bacteria for the appearance of disease symptoms in *Cyprinus carpio*

Pathogenic bacteria	Incubation period (in days)[a] of bacteria forthe appearance of disease symptoms
Gram negative	
Aeromonas hydrophila	3.34 ±0.33
Escherichia coli	7.64 ±0.57
Enterobacter aerogens	8.32 ±0.33
Shigella spp.	8.47 ±0.33
Pseudomonas fluorescens	6.75 ±0.68
Pseudomonas aeruginosa	8.13 ±0.33
Gram positive	
Micrococcus luteus	7.00 ±0.33
Staphylococcus aureus	5.07 ±0.57
CD Value(P < 0.05)	0.24

a = Mean ± S.E.; N = 30 (10 fish x 3 replications)

Table 2: Longevity of *Cyprinus carpio* injected with different pathogenic bacteria

Pathogenic bacteria Gram negative	Longevity (in days)[a]
Aeromonas hydrophila	8.66± 0.33
Escherichia coli	12.14 ± 0.57
Enterobacter aerogens	15.52± 0.33
Shigella spp.	16.37± 0.33
Pseudomonas fluorescens	11.53± 1.20
Pseudomonas aeruginosa	13.70 ± 0.57
Gram positive	
Micrococcus luteus	13.25 ±0.33
Staphylococcus aureus	10.38 ±0.57
CD (P < 0.05)	0.28

a = Mean ± S.E.; N = 30 (10 fish x 3 replications)

pathogenic bacteria. The fish injected with *A. hydrophila* survived for about 8 days post inoculation. The *A. hydrophila* was the most pathogenic bacterium among the isolated strains. *P. fluorescens* and *S. aureus* was also quite pathogenic as fish could live just for about 10-11 days post inoculation and mortality observed was 80.25% (Table 2). Among pathogenic bacteria maximum longevity was seen in *Shigella spp.* wherein fish survived for about 16 days. This was followed by fish injected with *E. aerogens* where longevity observed was about 15 days.

Antibacterial activity shown by earthworm species against isolated pathogenic bacteria.

***In vitro* antagonism test**

The earthworm species i.e. *Eudrilus eugineae, was* tested for their antagonistic behavior towards pathogenic bacterial strains using well diffusion technique. The results of the in *vitro* antagonism tests are presented in Table 3.

The maximum values of zone of inhibition against the fish bacterial pathogens were given by *Eudrilus eugineae* in the composition of 1:1(Extract: Ethanol)followed bypure extract and 1:2 (Extract: Ethanol).The mean values of the zone of inhibition observed for *Eudrilus eugeneia* against isolated pathogenic bacteria are shown in Table 3 (Fig. 1-8).

The maximum zone of inhibition observed for *Eudrilus eugeneia* was 21.00 mm against *A. hydrophila* and the minimum was 14.00 mm against *Shigell sp.* in gram negative bacteria. In case of gram positive bacteria the maximum value observed was 19.66 mm against *S. aureus*.

DISCUSSION

The present investigation began with screening of antibacterial activity of different earthworm species against isolated bacterial strains from healthy and diseased fish. 6 gram negative and 2 gram positive bacterial strains were isolated from fish, *Cyprinus carpio*. The number of bacterial isolates can be correlated with studies of various scientists who reported that intestinal micro flora of aquatic animals consists mainly of Gram-negative aerobic, obligate anaerobic and facultative anaerobic bacteria, the composition of which may change with environmental stresses (Ringo and Strøm, 1994; Ringo *et al*., 1997; Kennedy *et al*., 1998), diet (Munro *et al*., 1993; Douillet and Langdon, 1994; Gildberg *et al*., 1995;) and fish age (Bergh *et al*., 1994; Prayitno and Latchford, 1995; Olafsen, 2001). The isolated bacterial strains belonged to *Aeromonas* spp, *Pseudomonas* spp. and members of the family Enterobacteriaceae (Anand *et al*., 2011; Hassan *et al*., 2011; Satish *et al*., 2013). These bacteria

dominate micro flora of freshwater species (Ugajin, 1979; Sugita *et al*., 1988; Sakata, 1990; Sugita *et al*., 1991; Ringo *et al*., 1995). The motile aeromonas group, especially *A. hydrophila*, affects a wide variety of freshwater fish species and occasionally marine fish (Larsen and Jensen, 1977). *A. hydrophila* was found to be most pathogenic to fish as it took the least time to show the disease symptoms in fish. Harikrishnan *et al*. (2003) observed the pathogenicity induced by *A. hydrophila* when injected @10^8cfu/ml in common carp, *Cyprinus carpio* and found hemorrhagic spot at the site of injection on the 7th day, whereas in the present study the lesions appeared on the 4th day of the injection in the fish *Cyprinus carpio*. The prophylactic and therapeutic control of the bacterial diseases is based on oral administration of antibiotics. However, such treatment may cause the development of resistant bacteria (Aoki *et al*., 1985), yield residues in fish and introduce potential hazard to public health and to the environment. A new approach method, that is gaining acceptance within the industry, is the use of earthworm extract to control potential pathogens.

In the present study *in vitro* antagonism test following well diffusion assay was conducted to determine the zone of inhibition of earthworm extract against various fish pathogens. The maximum values of zone of inhibition against the fish bacterial pathogens were given by earthworm extract: ethanol (1:1) followed by pure extract and earthworm extract: ethanol (1:2) among all the composition used. The results of the study can be associated to study of Vasanthi *et al*. (2013) who worked onantimicrobial activity of earthworm paste *Eudrilus eugeniae* and found that **e**arthworm paste at a dose of 100μL was able to inhibit the growth of bacteria of *S. aureus* at a maximum level compared to other bacteria; the growth of fungal *Candida albicans* was much inhibited. Kathireswari *et al*. (2014) observed the similar results and revealed that coelomic fluid of earthworms *Lampito mauritii* and *Megascolex konkanensis* have high antimicrobial activity against *A. hydrophila, Bacillus subtillus, Vibrio para haemolyticus*.

Similar results were observed by Mathur *et al*. (2010) who

Figure 1: Zone of inhibition against *A. hydrophila*.

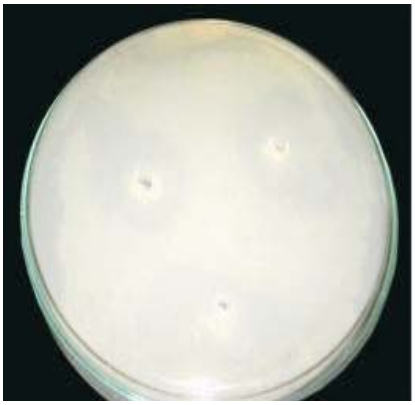

Figure 2: Zone of inhibition against *E. aerogens*

Table 3: Inhibition zones (in mm) of *Eudrilus eugineae* against different pathogenic bacteria.

Bacterial strains	Pure Extract	Extract:Ethanol1:1	Extract:Ethanol 1:2
Aeromonas hydrophila	20.33 + 0.33	21.00 + 0.57	18.66 + 0.33
Staphylococcus aureus	17.66 + 0.33	19.66 + 0.33	14.66 + 0.66
Pseudomonas aeruginosa	17.33 + 0.33	17.33 + 0.33	15.33 + 0.33
Pseudomonas floresecens	18.33 + 0.33	19.33 + 0.33	15.00 + 0.57
Micrococcus luteus	13.00 + 0.57	15.00 + 0.57	12.66 + 0.33
Escherichia coli	19.00 + 0.57	20.66 + 0.33	15.33 + 0.33
Enterobacteraerogens	17.66 + 0.33	18.33 + 0.33	14.66 + 0.33
Shigella spp.	11.33 + 0.88	14.00 + 0.57	9.00 + 0.57
C.D. (Pd″0.05)	1.51	1.33	1.38

a = Mean ± S.E.

Figure 3: Zone of inhibition against *E. coli*

Figure 4: Zone of inhibition against *P. fluorescens*

Figure 5: Zone of inhibition against *S. aureus*

Figure 6: Zone of inhibition against *P. aeruginosa*

studied antimicrobial activity of earthworm extracts of various solvents and found that found that 95% ethanol extract of earthworm was potent antibacterial agent against *Streptococcus* pyogens and antifungal agent against *Candida albicans*. Istiqumah *et al*. (2012) studied to determine the inhibitory of earthworm (*Lumbricus rubellus*) extract (ECT) and encapsulated earthworm extract (ECT-t) as poultry feed additive against some pathogenic bacteria and found that i*n vitro* antibacterial activity was performed using dilution method against *Escherichia coli, Staphylococcus aureus, Salmonella pullorum* and P*seudomonas aeruginosa*. Elif Ozlem and Cotuk (2008) worked on antibacterial and haemolytic activity of the coelomic fluid of *Dendrobaen aveneta* (Oligochaeta, Lumbricidae) living in different localities and concluded that coelomic fluid of Beykoz earthworms was a potential agent, which can be used as an alternative drug, since this coelomic fluid was effective against bacteria. The present study indicates that the earthworm extract has good antibacterial potency. Similar results were reported by Shobha and Kale (2008) who found that coelomic fluid has antibacterial activity against bacteria. Bhorgin and Uma (2014) also found that 95% ethanol extract of earthworm was potent antimicrobial agent against

Figure 7: Zone of inhibition against *M. luteus*

Figure 8: Zone of inhibition against *Shigella spp.*

A. hydrophila and antifungal agent against *C. albicans*. Based on the living habitats of earthworms, it is rational to think that there are some bioactive compounds that showed effective antimicrobial agents in earthworm's skin. Cho *et al*. (1998) identified the first antimicrobial peptide (lumbricin I) from the earthworm, *Lumbricus rubellus*. Lumbricin I is considered as a proline-rich antimicrobial peptide containing 62 amino acids including proline (15%) with a molecular weight of 7231 Da. Lumbricin I showed antimicrobial activity *in vitro* against a broad spectrum of microorganisms without hemolytic activity. Engelmann *et al*. (2004) and Balamurugan *et al*. (2008) found that earthworm coelomic fluid contains biologically active molecules and leukocytes that participate in phagocytosis, encapsulation and killing of HeLa, HEp-2, PC-12 and PA317 cells *in vitro*.

In conclusion, it can be stated that earthworm extract can reduce the incidence and duration of diseases. The application of earthworm extract in aquaculture shows promise but needs considerable efforts of research.

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