

Original Research Article

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Antibacterial Effects of *Eisenia fetida*, Earthworm Extract against Pathogenic Bacteria Isolated from *Cyprinus carpio*

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ABSTRACT

In the present investigations, whole body extract of earthworm, *Eisenia fetida* was tested for antibacterial activity against fish bacterial pathogens. The bacteria were isolated from different tissues of infected common carp, *Cyprinus carpio* and identified by gram staining method. A total of eight pathogenic bacteria were isolated. Antimicrobial activity of the extract was studied under *in vitro* conditions by well diffusion assay. The extract of *Eisenia fetida* showed antibacterial activity against all the pathogenic bacteria and the maximum zone of inhibition was observed against *A. hydrophila*. Histological investigations had revealed that feeding the fish on a diet prepared by incorporating earthworm extract resulted in an increase in intestinal folds while injecting the fish with *A. hydrophila* resulted in degeneration and desquamation of intestinal epithelium feeding on supplementary diet. When a healthy carp was injected with pathogenic bacterium and also fed on a diet prepared in earthworm extract, it resulted in a significant ($P < 0.05$) increase in intestinal folds, intestinal microvilli, increase in concentration of melano-macrophage centre and also haemopoietic tissue in the kidneys. From these studies, it was concluded that the earthworm extract possesses very strong antibacterial properties which could be employed in treating bacterial infections in fishes, leading to the availability of antibiotic free fishes to the consumers.

Keywords

Bacteria, Common carp, *Eisenia fetida*, Antibacterial activity, Intestine, Kidney

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Introduction

Fishery is an important sector in India as it provides employment to millions of people and also contributes to the food security of the country. Massive fish mortalities have been reported from different countries due to bacterial, fungal, viral and protozoan infections/diseases that results in heavy economic losses. A variety of Bacterial

infections pose significant threats to successful fish production throughout the world.

Diseases as well as environmental pollution play an important role in aquaculture growth. Bacterial diseases outbreaks impose a significant constraint in fish and shellfish production (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). Use of antimicrobial drugs in aquaculture has led to the emergence of many antibiotic resistant bacteria (Schwarz *et al.*, 2001; Akinbowale *et al.*, 2006).

Biological active compounds extracted from earthworms has traditionally been used by indigenous people throughout the world, more particularly in Asia, including India, Myanmar, China, Korea and Vietnam (Ranganathan, 2006). Since long time, earthworms have been used as a therapeutic drug source in China and other parts of the Far East countries (Ismail, 2005). Studies have shown the antipyretic, antispasmodic, detoxic, diuretic, antihypertensive, antiallergic, anticoagulative, antiasthmatic, spermatocidal, antioxidative, antimicrobial, anticancer, antiulceral and anti-inflammatory activities particularly of biologically active compounds isolated from earthworms (Hrzenjak *et al.*, 1998; Wang *et al.*, 2007; John and Packialakshmi, 2007; Balamurugan *et al.*, 2009; Cooper and Balamurugan, 2010; Ansari and Sitaram, 2010). Prakash *et al.*, (2007) and Balamurugan *et al.*, (2007) have reported the presence of anti-ulceral and anti-oxidative properties in the paste of *Lampito mauritii*. Histopathological studies are recognized as biomarkers in the evaluation of the health of fish exposed to contaminants/pathogenic microbes/metazoan parasites, both in the laboratory (Thophon *et al.*, 2003) and field studies. The histopathological studies on various target organs like intestine, gill, kidney and liver are useful to assess the damage to fish and animal health.

A number of studies have been carried out by researchers across the globe for assessing and evaluating the antibacterial activity of earthworm extract in many animal species.

However, not many 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

Collection of samples

Both healthy and infected specimens of *Cyprinus carpio* were obtained from fish farms in Hisar (Haryana) and brought to the Fish Biotechnology Laboratory of the Department of Zoology, CCS Haryana Agricultural University, Hisar (India). The earthworm, *Eisenia fetida* were collected from the vermicomposting Unit of the Department of Zoology, Chaudhary Charan Singh Haryana Agricultural University, Hisar.

Isolation of bacteria

Tissue samples from skin lesions, gills and intestine were dissected out from both healthy and infected common carp. Tissues were homogenized in Phosphate buffer saline (PBS) and then centrifuged at 12000 rpm for 10 min. Supernatant was spread over the nutrient agar (NA) medium under laminar flow. Plates were incubated in BOD incubator maintained at $30 \pm 2^\circ$ C for 18-24 hours. Growth on NA plate was observed after 18-24 h. Pure colonies of bacteria were isolated by further sub-culturing of single colonies on NA plates (Gerhardt *et al.*, 1994).

Identification of bacteria

Isolated pure cultures of bacteria were subjected to biochemical tests (primary and secondary tests) for identification as following Krieg and Holt (1984) in "Bergey's Manual of

Determinative Bacteriology". Identification of bacteria was done with the help of computer program, PIBWin (Website: www.soton.ac.in.uk/tnb/pib.htm).

***In vivo* pathogenicity testing**

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

Preparation of earthworm extract

Sexually mature clitellated earthworms (*Eisenia fetida*) were washed with running tap water and then fed with wet blotting paper for 18-20 hours for gut clearance. These worms were again washed with distilled water. In order to kill the worms they were kept in plastic trough covered with polythene in sunlight for three days.

Mucus and coelomic fluid that oozed out 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 (Balamurugan *et al.*, 2007). After dried the paste in incubator, it was mixed with nuclease free water and centrifuged the whole mixture at 12000 rpm for 10 min. The supernatant was used as aqueous earthworm extract. The 95% ethanolic extract of earthworm were also prepared using aqueous extract in different combination.

Preparation of feed

During the experiment the fingerlings of common carp were acclimatized for 15 days under laboratory conditions and fed with the

normal diet. After 15 days the experiments were conducted by feeding fish with normal diet and diet containing earthworm extract. The normal diet was prepared by using the following composition:

The extract was weighed on electronic balance @ 1.0%, 1.5% and 2.0% of the total feed gradients. Feed ingredients were mixed with the paste/ extract of earthworms and then the pellets of feed were prepared. All ingredients were mixed well and then dough was made by wetting with sterilized double distilled water. Pellets were obtained by pelletizer and then dried in BOD at 55 °C temperature.

The experiment was conducted in the Aquarium Room of the department. The aquaria were supplied with well aerated tap water and each tub was supplied by compressed air via-stone from aerator. Fresh tap water was stored in cuboidal tanks (of 400-500 liters capacity) for 24 hours in order to dechlorinate the water. Each tub was cleaned every alternate day by siphoning fish feces and remaining feed.

The feed, normal or supplemented with earthworm extract was given at the rate of 2% of body weight.

Antibacterial well diffusion assay

The clear zone of inhibition was measured using a centimeter scale and the observations were recorded following Gram and Melchiorson (1996).

Histopathological examination

Tissue samples obtained from intestine and kidney of experimental fish were fixed in Bouin's fixative for 24 h. Following usual procedures, paraffin embedded sections were cut at 4-6µm thickness and stained in Ehrlich Hematoxylin-Eosin.

Statistical analysis

Data were analyzed using completely randomized design (CRD) following Snedecor and Cochran (1989).

Results and Discussion

Eight bacterial strains were reported and identified from *Cyprinus carpio* (Table 1). This is in agreement with the findings of other workers who have also shown that intestinal micro flora of aquatic animals consists of mainly Gram-negative aerobic, obligate anaerobic and facultative anaerobic bacteria and the composition of which may vary with environmental stresses (Ringo *et al.*, 1997; Kennedy *et al.*, 1998; Olafsen, 2001). Various bacterial strains that have been isolated from aquatic animals belonged to *Aeromonas* and *Pseudomonas* spp. and also some species of the family Enterobacteriaceae (Anand *et al.*, 2011; Hassan *et al.*, 2011; Satish *et al.*, 2013).

The longevity of fish infected with different pathogenic bacteria is shown in table 2. The *A. hydrophila* appeared to be the most pathogenic bacterium among the isolated strains. As the fish injected with *A. hydrophila* survived for about 8 days post inoculation. *Pseudomonas fluorescens* and *Staphylococcus aureus* were also quite pathogenic as fish could survive just for about 10-11 days post inoculation and mortality was 80.25% (Table 2). *A. hydrophila* was found to be most pathogenic to fish as it took the least time to show the disease symptoms in fish (Sarkar and Rashid, 2012).

According to Harikrishnan *et al.*, (2003) pathogenicity was induced by *A. hydrophila* when injected @ 10^8 cfu/ml on 7th day of injection and also reported hemorrhagic spots at the site of injection in common carp, whereas, in the present studies, similar symptoms appeared on the 4th day of the injection in common carp.

Antimicrobial well diffusion assay

The results of the *in vitro* antagonism tests are shown in table 3. Maximum values of zone of inhibition against the fish bacterial pathogens were shown by *Eisenia fetida* in the composition of 1:1 (Extract: Ethanol).

The mean values of the zone of inhibition observed for *Eisenia fetida* against the Gram -ve bacteria was 24.00 mm for *A. hydrophila* and against Gram+ve bacteria was 21.33 mm for *S. aureus* (Table 3, Fig. 1-6).

Similar to our findings, Vasanthi *et al.*, (2013) found that extract of *Eudrilus eugeniae* at a dose of 100 μ l was able to inhibit the growth of *S. aureus* at a maximum level compared to other bacteria.

Present studies have revealed that earthworm extract possesses good antibacterial potency and these results are similar to other findings reported by other workers who have observed that coelomic fluid has antibacterial activity against bacteria (Mathur *et al.*, 2010; Shobha and Kale, 2008; Kathireswari *et al.*, 2014; Bansal *et al.*, 2015, 2016).

Bhorgin and Uma (2014) also found that 95% ethanol extract of earthworm showed potent antimicrobial agent against *A. hydrophila* and antifungal activity against *C. albicans*.

Histopathological study

The exposure of *Cyprinus carpio* to bacterial strains has revealed clear differences between the effect of pathogenic bacterium (*A. hydrophila*) infection and earthworm extract on treated groups.

No differences were observed in the histology of the foregut of the common carp treated with earthworm extract as it was similar to control fish as both had intact epithelial barrier.

Table.1 List of bacteria isolated from infected/diseased fish

Selective Media	Confirmation of bacteria
Antibiotic Assay Medium No. 38	<i>Pseudomonas aeruginosa</i>
Antibiotic Assay Medium. C	<i>Staphylococcus aureus</i>
EMB medium	<i>Enterobacter aerogens</i>
Hugh Leifson glucose medium	<i>Micrococcus luteus</i>
Pseudomonas agar F Base	<i>Pseudomonas fluorescence</i>
Rimler-Shotts Medium	<i>Aeromonas hydrophila</i>
XLD (Xylose deoxycholate agar) EMB Agar (Eosin Methylene Blue Agar)	<i>Shigella spp.</i>

Table.2 Pathogenicity of bacteria and longevity of *Cyprinus carpio*

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

All values are Mean ± S.E of mean; N=30 (10 fish x 3 replications)

Table.3 Inhibition zones (in mm) of *Eisenia fetida* against different pathogenic bacteria.

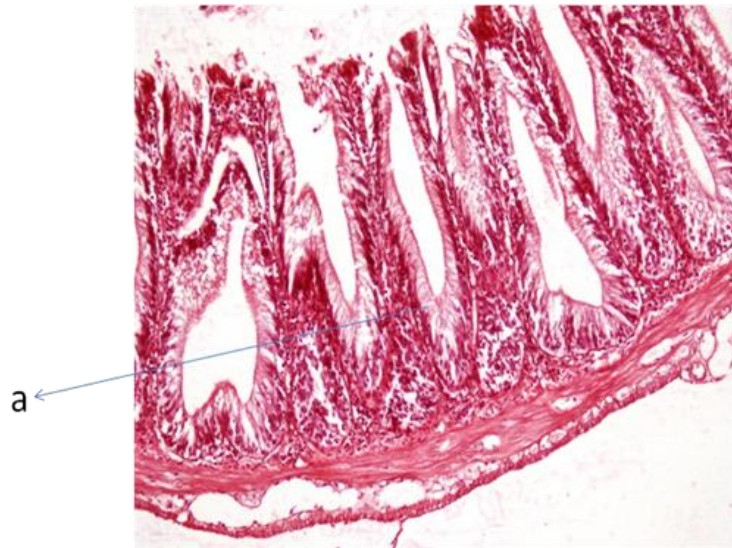
Bacterial strains	<i>Eisenia fetida</i>		
	Pure Aqueous Extract	Aqueous Extract: Ethanol 1:1	Aqueous Extract: Ethanol 1:2
<i>Aeromonas hydrophila</i>	21.33±0.33	24.00±0.66	21.33±0.57
<i>Staphylococcus aureus</i>	19.00±0.57	21.33±0.57	18.00±0.33
<i>Pseudomonas aeruginosa</i>	15.66±0.33	18.00±0.33	14.66±0.57
<i>Pseudomonas florescens</i>	18.33±0.88	20.33±0.57	17.00±0.33
<i>Micrococcus luteus</i>	14.00±0.57	16.00±0.66	11.33±0.57
<i>Escherichia coli</i>	19.33±0.66	21.00±0.33	17.66±0.57
<i>Enterobacter aerogens</i>	18.33±0.33	18.66±0.66	15.33±0.33
<i>Shigella spp.</i>	11.66±0.33	15.33±0.33	11.66±0.33

All values are Mean ± S.E of mean; N=30 (10 fish x 3 replications)

Preparation of feed

Components	Amount in %
Soya Flour	16.5
Ground nut oil cake (GNOC)	16.5
Rice Bran	17.0
Fish meal	16.5
Sesame oil cake	16.5
Tapioca flour	16.5
MPA	0.5

Fig.1 Histological study of intestine in control group (40X)



a) Intact or normal intestinal epithelium

Fig.2 (a and b). Histological changes in intestine in fish inoculated with pathogenic bacterium (10X). a) Fusion of microvilli of intestine along with infiltration of MNC's

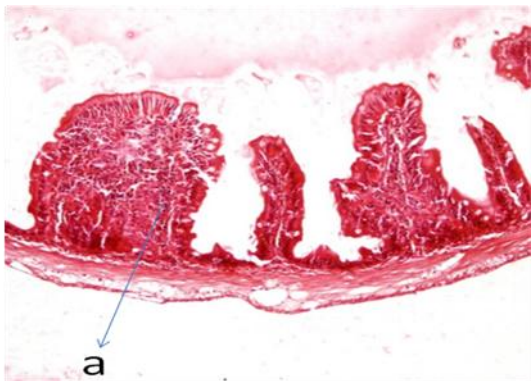


Fig.2 (a)

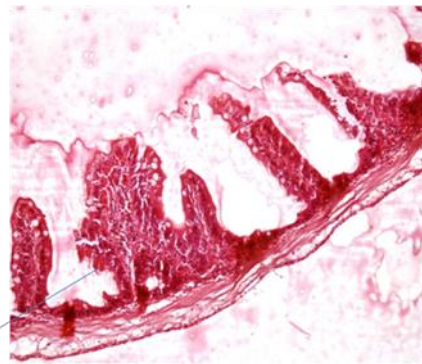


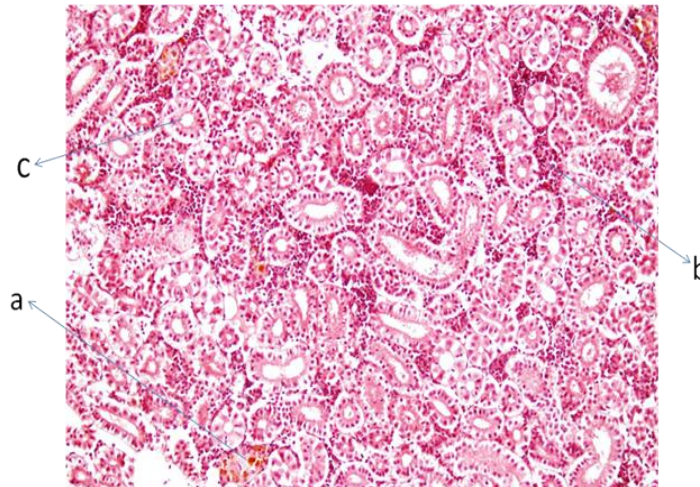
Fig.2 (b)

Fig.3 Histological changes in intestine in fish fed with Earthworm extract (40X)



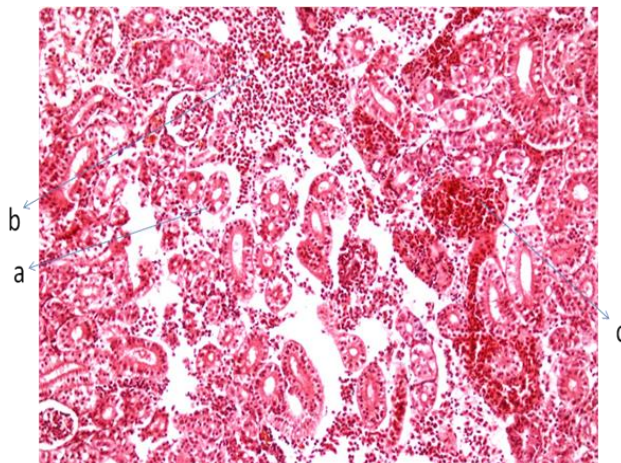
a) Increased intestinal folds

Fig.4 Histological study of kidney in control group (10X)



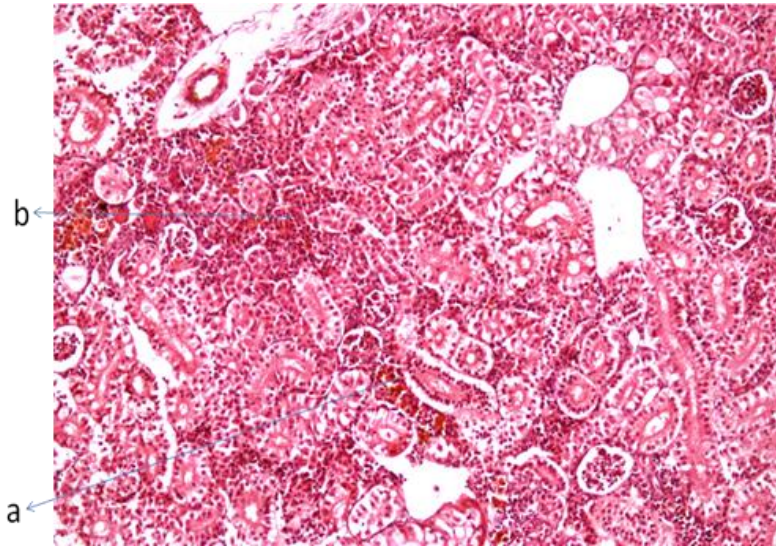
a) Melano-macrophage center, b) Haemopoietic tissue, c) Tubules of kidney

Fig.5 Histological changes in kidney in fish inoculated with pathogenic bacterium (10X)



a) Vacuolation and degeneration of tubule epithelium, b) Infiltration of MNC's c) Haemorrhage

Fig.6 Histological changes in kidney in fish fed with earthworm extract (10X)



a) Increased Melano-macrophage centres, b) Haemopoietic tissue c) Mild hyaline tubular casts of kidney

Fish infected with *A. hydrophila* showed severe enteritis in intestine characterized by degeneration and desquamation of epithelium in villi along with infiltration of mono-nuclear cells (Fig. 2). Similarly, severe changes were also observed in the histology of kidney where severe vacuolation and degeneration of tubular epithelium along with infiltration of mono-nuclear cells were observed. Hemorrhages were also observed in the parenchyma of the kidney (Fig. 5). Exposure of the foregut to the pathogenic bacterium (*A. hydrophila*) resulted in various damaging effects i.e. altered microvilli and protruding epithelial cells sloughing into the lumen, and the presence of cell debris in the gut lumen.

In the treatment, where the fish were injected with bacteria and fed on a diet incorporated with earthworm extract showed slight degeneration and desquamation of the intestinal villi epithelium and infiltration of mono-nuclear cells in intestine (Fig. 3). The increase in number of folds in the intestine caused by earthworm extract is definitely a beneficial effect on the host. In kidney, increased concentration of melano macrophage centre, haemopoietic tissue with

presence of mild hyaline tubular casts (Fig. 6) were observed. No histopathological changes were observed in intestine and kidney in the control group (Fig. 1 and 4). Similar findings were observed by Fatma Mohammad (2009), who worked on histopathological study of *Tilapia zilli* and *S. vulgaris* also observed that most common lesions in liver were vacuolar degeneration and kidney showed severe degenerative and necrotic changes in renal tubules with focal areas of necrosis and haemorrhage, haemolysis and vacuolar degeneration of renal tubules. In the kidney, necrosis of tubular epithelium in kidney with focal lymphocyte infiltration was considered to be associated with interstitial nephritis which seemed to be parallel with previous studies (Errer, 1981; Roberts, 2001; Hassan *et al.*, 2008).

Present studies had clearly revealed that the earthworm extract is very effective in controlling the fish diseases in order to improve their health status. The present study further showed that fish treated with earthworm extract showed an improvement in the histopathology of common carp. The exposure of *Cyprinus carpio* to bacterial

strains has revealed clear differences between the effect of pathogenic bacterium (*A. hydrophila*) infection and earthworm extract on treated groups. 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. A new approach method, that is gaining acceptance within the industry, is the use of earthworm extract to control potential pathogens.

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