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Comparison of Antibacterial Activities of Coelomic Fluid of Local Species of Earthworms of Gulbarga

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ABSTRACT

Coelomic fluid of earthworm is having bactericidal activity. It was our interest to know the bactericidal activity of the coelomic fluid of Polypheretima elongata,Perionyx sansibaricus and Dichogaster bolaui against pathogenic bacteria like Escherichia coli, Staphylococcus aureus, Pseudomonas, Proteus, and Bacillus. Polypheretima elongata, Perionyx sansibaricus and Dichogaster bolaui are local species of earthworms present in Gulbarga. Bactericidal activity coelomic fluid of the earthworm was tested among the three species and in comprision with the commercial antibiotics available in the market. The batricidal activity of the coelomic fliud of the earthworm was determined by the zone of inhibition by coelomic fluid of earthworm Polypheretima elongata, Perionyx sansibaricus and Dichogaster bolaui against pathogenic bacterial strains. Our results show that the zone of inhibition varies from species to species, and it is species specific to particular bacteria. It is clear that antibacterial activity is exhibited at different levels by coelomic fluid of Polypheretima elongata,Perionyx sansibaricus and Dichogaster bolaui.The coelomic fliud of Polypheretima elongata exhibited highest bactericidal activity against Escherichia coli and Staphylococcus aureus. Perionyx sansibaricus has highest bactericidal activity against Proteus and Pseudomonas.Dichogaster bolaui against Stphylococcus aureus and Bacillus

KEYWORDS : Earthworm, Bactericidal activity, Coelomic fluid.

1. Introduction

Invertebrates exhibit different immune mechanisms against environmental pathogens. In earthworms, coelomocytes (leucocytes) located in coelomic cavity are responsible for innate cellular immune functions such as phagocytosis and encapsulation against parasites and pathogens. The coelomocyte population has been divided into different subpopulations. The coelomocytes possess immuno-defense related biological functions. The effector cells participate mainly in cellular mechanism but chloragocytes and the granulocytes of coelomocytes may produce humoral factors which may mediate the cellular and humoral responses as well. Microbial killing results from the combined action of the phagocytic process with humoral immune factors such as agglutinin (viz., lactin), lysosomal enzymes (viz., acid phosphatases) and various cytotoxic and antimicrobial molecules.

Invertebrates have developed innate immune mechanism that detects pathogens by recognizing conserved molecular patterns. Molecules responsible for the recognition of foreign material have been named as pattern recognition proteins (PRPs) (Medzhitov and Janeway, 1997) because the host's primitive effector cells would recognize molecular pattern rather than particular structure of the invading microorganisms. Examples of pathogen–associated molecules, which are not found in the multicelluar organisms, are lipopolysaccharides (LPS) or peptidoglycans from bacterial cell walls β-1, 3-glucan of fungal cell walls and double stranded RNA of viruses.

Immune mechanism of earthworms includes both cellular and humoral components. Earthworm coelomocytes respond to the presence of pathogens by phagocytosis, encapsulation/brown body formation and N K cells activity (Cooper *et al*., 2001). Humoral components include lectin, antimicrobial peptides, pore forming proteins, phenoloxidases and proteases. They include sequestration of antigenic material by agglutination, cytotoxicity and antibacterial activity. Cytolisin of *Eisenia fetida* coelomic fluid, named eiseniapore, was found to cause pore like structure at the target membranes. Formation of pore proteins is the result of oligomarization of eiseniapore monomers (Lange *et al*., 1999).

There are many investigations on the presence of antibacterial substances in coelomic fluid of earthworms since bacterolytic molecules were identified as lysozyme like molecules, active only against gram positive bacteria (Jolles and Zuili, 1960; Scherbert and Messner, 1997; Lassegues, 1986). The coelomic fluid of earthworm, Eisenia fetida (Oligochaeta: Lumbricidae) was demonstrated to possess an antibacterial activity directed against earthworm pathogenic bacteria –namely: Gram negative Aeromonas hydrophila and Gram-positive Bacillus megaterium by Valembois et al. (1992).

Our present study is aimed to know the presence of different types of coelomocytes and antibacterial activity of coelomic fluid (crude form) of the tropical earthworms Polypheretima elongata, Perionyx sansibaricus and Dichogaster bolaui on pathogenic bacteria like Escherichia coli, Staphylococcus aureus, Pseudomonas, Proteus, and Bacillus. This antibacterial activity of the coelomic is fluid compared with that of commercially available antibiotics like Streptomycin, Norfloxacin, Ciprofloxacin, Gentamicin and Amoxillin.

2 MATERIALS AND METHODS:

Bactericidal studies

The bacterial strains used for determining antibacterial activity are Escherichia coli, Staphylococcus aureus, Pseudomonas, Proteus and Bacillus which were obtained from Department of Microbiology, Gulbarga University, Gulbarga. The nutrient agar media was prepared in the laboratory by adding agar to the nutrient broth (Hi-Media make). Whatman no. 1 filter paper was used to prepare the discs. Commercially available antibiotic discs (Streptomycin, Norfloxacin, Ciprofloxacin, Gentamicin and Amoxillin) were purchased from Hi-media. Sterile swabs were purchased from local medical distributors. Nutrient agar media was purchased from Hi-media.

Method of extraction of coelomic fluid for antibacterial activities:

Earthworms were washed, kept on soft paper to absorb surface body moisture and then transferred to Petri dish, excited with 6 volt electrical stimulation, which induced them to extrude coelomic fluid through epidermal dorsal pores. The fluid extracted was collected in the small tubes and used for antibacterial tests. Sterilized blotting paper (about 6 mm diameter), free from any antibacterial activity, were impregnated with 20 µl of the fraction to be tested and placed on agar dishes inoculated with bacterial strain.

Disk method:

The target bacterial species were separately cultured in the nutrient broths to yield 10-7 cfu/ml and 18-24 hr cultures were separately spread plated on nutrient agar plates. Then, the 6 mm paper disks impregnated with 20 µl coelomic fluid fraction and incubated upright at 37^{0}C in an incubator for 24 h. The bactericidal activity of the fractions was measured in terms of mm zone of inhibition. Antibacterial activity of the coelomic fluid of the Polypheretima elongata, Perionyx sansibaricus and Dichogaster bolaui were compared with the commercial antibiotics available in the market viz., Streptomycin, Norfloxacin, Ciprofloxacin, Gentamicin and Amoxillin.

3 RESULTS:

Bactericidal activity:

5.3.2.1 *Polypheretima elongata*

The influence of coelomic fluid of Polypheretima elongata on the in vitro growth of bacterial cultures was evaluated against pathogenic bacteria such as Escherichia coli, Proteus, Pseudomonas, Staphylococcus aureus and Bacillus. The best inhibitory effect of coelomic fluid

of Polypheretima elongata on the growth of Escherichia coli, Proteus and Staphylococcus aureus was seen to be 9 mm to 10 mm zone of inhibition. The inhibitory effect is comparatively less against Pseudomonas, and Bacillus, exhibiting 5 mm and 7 mm inhibitory zones in Pseudomonas and Bacillus respectively (Table – 5.2, Plate 5.1 to 5.5).

Table – 5.2: Zone of inhibition (antibacterial activity) obtained by the coelomic fluid of Polypheretima elongata and commercial antibiotics Amoxillin, Streptomycin, Norfloxacin, Ciprofloxacin and Gentamicin against the growth of pathogenic bacteria like Escherichia coli, Staphylococcus aureus, Pseudomonas, Proteus and Bacillus

Bacteria used	Zone of inhibition (mm) P. elongata	Amoxillin (mm)	Streptomycin (mm)	Norfloxacin (mm)	Ciprofloxacin (mm)	Gentamycin (mm)
Escherichia coli	9	12	24	2	25	15
Proteus	9	18	2	22	23	2
Pseudomonas	5	_	24	32	38	18
Staphylococcus aureus	10	14	22	25	34	24
Bacillus	07	_	3	3	33	19

Plate – 5.1: Zone of suppression impregnated with coelomic fluid of Polypheretima elongata on Escherichia coli



Plate – 5.2: Zone of suppression impregnated with coelomic fluid of Polypheretima elongata on Proteus

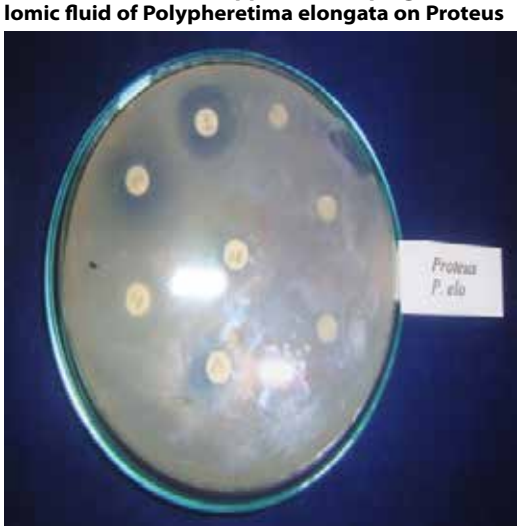


Plate – 5.3: Zone of suppression impregnated with coelomic fluid of Polypheretima elongata on Pseudomonas

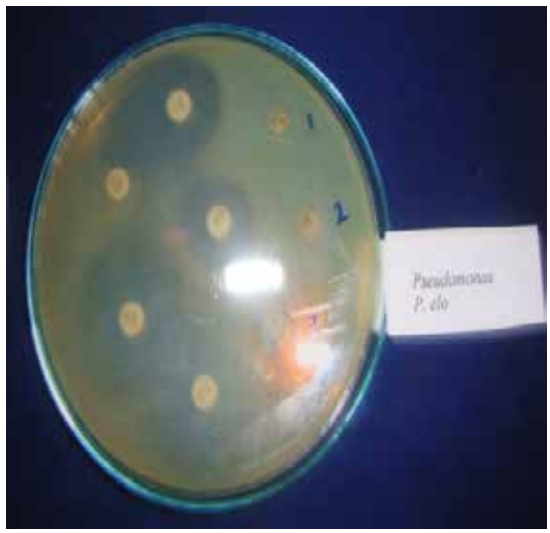
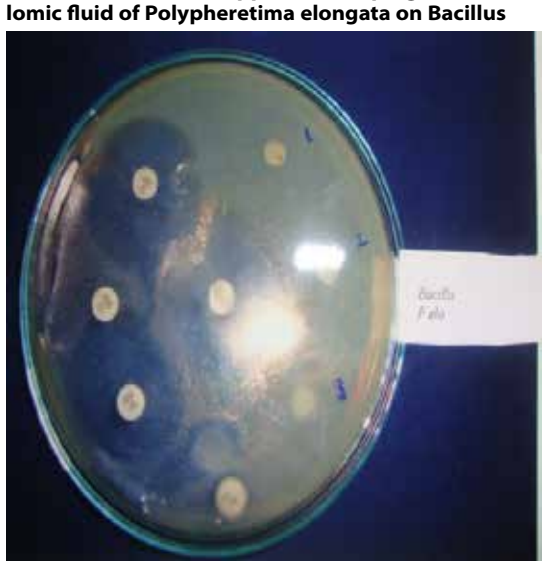


Plate – 5.4: Zone of suppression impregnated with coelomic fluid of Polypheretima elongata on Staphylococcus aureus



Plate – 5.5: Zone of suppression impregnated with coelomic fluid of Polypheretima elongata on Bacillus



5.3.2.2 Perionyx sansibaricus

The influence of coelomic fluid of Perionyx sansibaricus on the in vitro growth of bacterial culture was evaluated against Escherichia coli, Proteus, Pseudomonas, Staphylococcus aureus and Bacillus. The best inhibitory effect of coelomic fluid of Perionyx sansibaricus on the growth of Proteus is 16 mm and that on Pseudomonas 8 mm, where as the zone of inhibition is only 7 mm against Escherichia coli, Staphylococcus aureus and Bacillus (Table – 5.3, Plate 5.6 to 5.10).

5.3.2.3 Dichogaster bolaui

The influence on coelomic fluid of Dichogaster bolaui on the in vitro growth of bacterial culture was evaluated against pathogenic bacteria such as Escherichia coli, Proteus, Pseudomonas, Staphylococcus aureus and Bacillus. The best inhibitory effect of coelomic fluid of Dichogaster bolaui was observed against Escherichia coli, Staphylococcus aureus and Bacillus, 8 mm and 9 mm inhibitory zones being observed. This inhibitory zone is comparatively smaller against Proteus and Pseudomonas (only 5 mm to 6 mm) (Table – 5.4, Plate 5.11 to 5.15).

The above results indicate that the coelomic fluid of species of earthworm is species-specific in antibacterial activity (Graph 5.1).

This antibacterial activity of coelomic fluid of earthworm is compared with that of commercially available antibiotics such as Streptomycin, Norfloxacin, Ciprofloxacin, Gentamicin and Amoxillin; it shows slightly milder activity in many instances.

Table – 5.3: Zone of inhibition (antibacterial activity) obtained by the coelomic fluid of Perionyx sansibaricus and commercial antibiotics Amoxillin, Streptomycin, Norfloxacin, Ciprofloxacin and Gentamicin against the growth of pathogenic bacteria like Escherichia coli, Staphylococcus aureus, Pseudomonas, Proteus and Bacillus

Bacteria used	Zone of inhibition P. sansibaricus (mm)	Amoxillin (mm)	Streptomycin (mm)	Norfloxacin (mm)	Ciprofloxacin (mm)	Gentamicin (mm)
Escherichia coli	07	05	19	2	27	15
Proteus	16	09	19	21	22	15
Pseudomonas	8	12	1	25	1	2
Staphylococcus aureus	07	26	25	3	28	27
Bacillus	07	12	23	32	3	2

Plate – 5.6: Zone of suppression impregnated with coelomic fluid of Perionyx sansibaricus on Escherichia coli

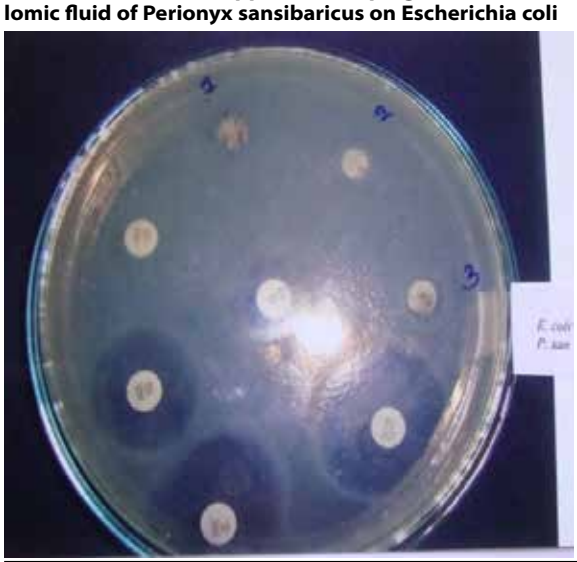


Plate – 5.7: Zone of suppression impreg nated with coelomic fluid of Perionyx sansibaricus on Proteus

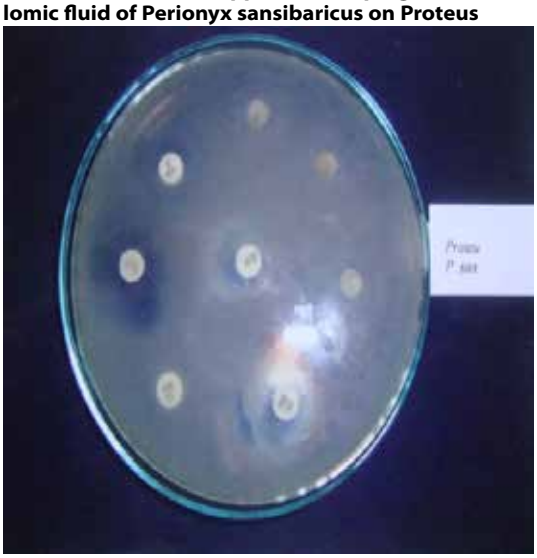


Plate – 5.8: Zone of suppression impreg nated with coelomic fluid of Perionyx sansibaricus on Pseudomonas

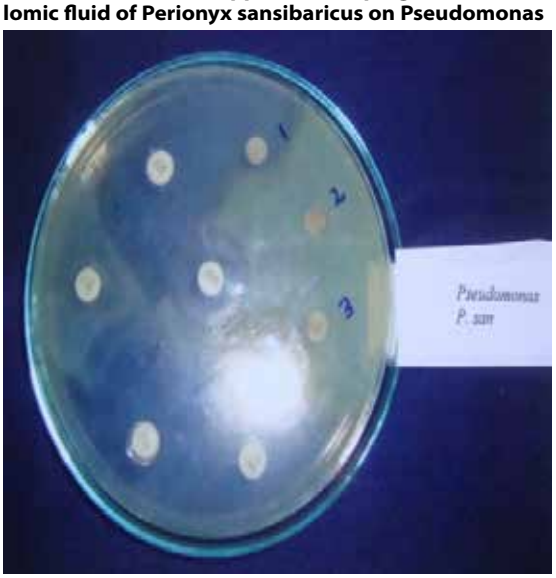


Plate – 5.9: Zone of suppression impreg nated with coelomic fluid of Perionyx sansibaricus on Staphylococcus aureus

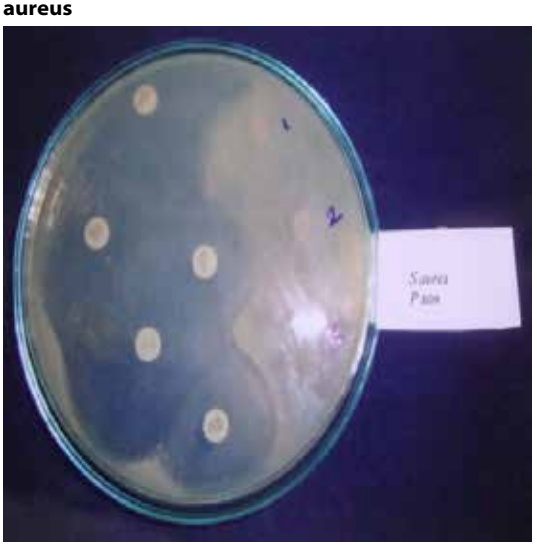


Plate – 5.10: Zone of suppression im pregnated with coelomic fluid of Perionyx sansibaricus on Bacillus

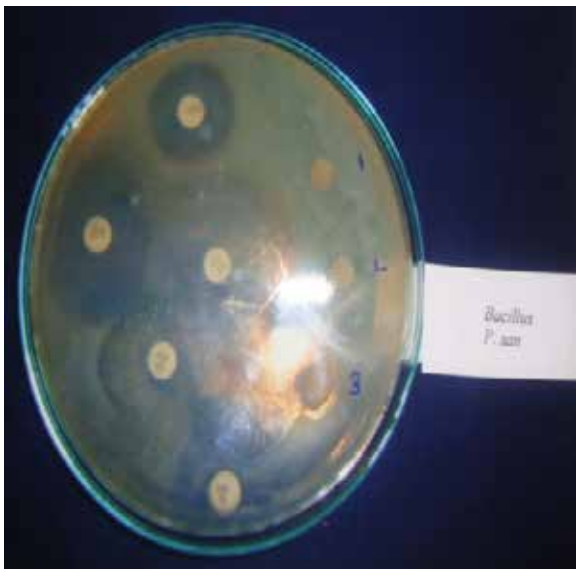


Table – 5.4: Zone of inhibition (antibacterial activity) obtained by the coelomic fluid of Dichogaster bolaui and commercial antibiotics Amoxillin Streptomycin, Norfloxacin, Ciprofloxacin and Gentamicin against the growth of pathogenic bacteria like Escherichia coli, Staphylococcus aureus, Pseudomonas, Proteus and Bacillus

Bacteria used	Zone of inhibition D. bolaui (mm)	Amoxillin (mm)	Streptomycin (mm)	Norfloxacin (mm)	Ciprofloxacin (mm)	Gentamicin (mm)
Escherichia coli	8	15	02	18	25	14
Proteus	6	05	28	2	22	2
Pseudomonas	5	_	25	3	26	19
Staphylococcus aureus	9	15	17	3	3	25
Bacillus	9	_	28	3	3	2

Plate – 5.11: Zone of suppression impregnated with coelomic fluid of Dichogaster bolaui on Escherichia coli



Plate – 5.12: Zone of suppression impregnated with coelomic fluid of Dichogaster bolaui on Proteus

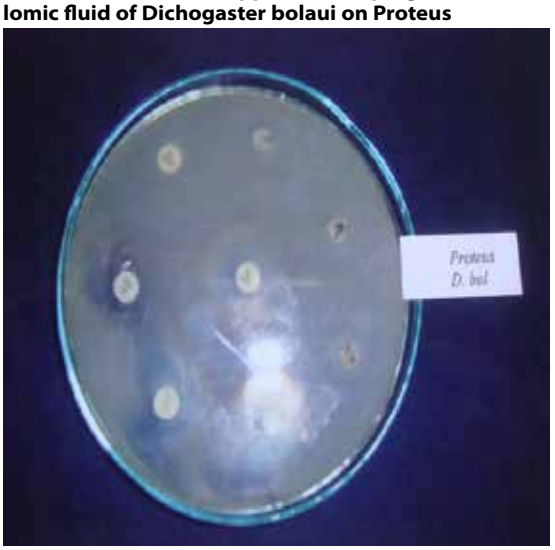


Plate – 5.13: Zone of suppression impregnated with coelomic fluid of Dichogaster bolaui on Pseudomonas

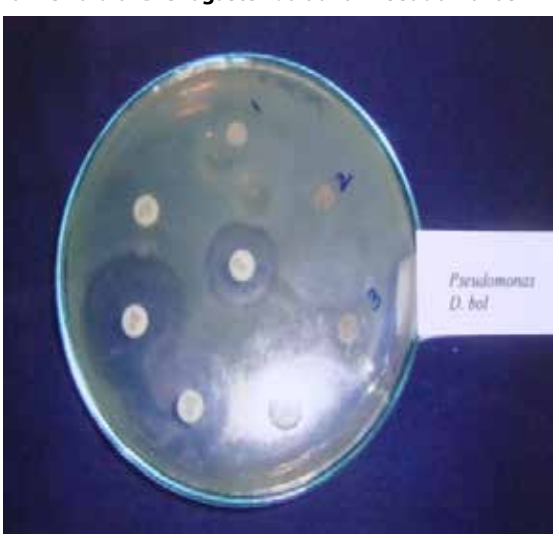


Plate – 5.14: Zone of suppression impregnated with coelomic fluid of Dichogaster bolaui on Staphylococcus aureus

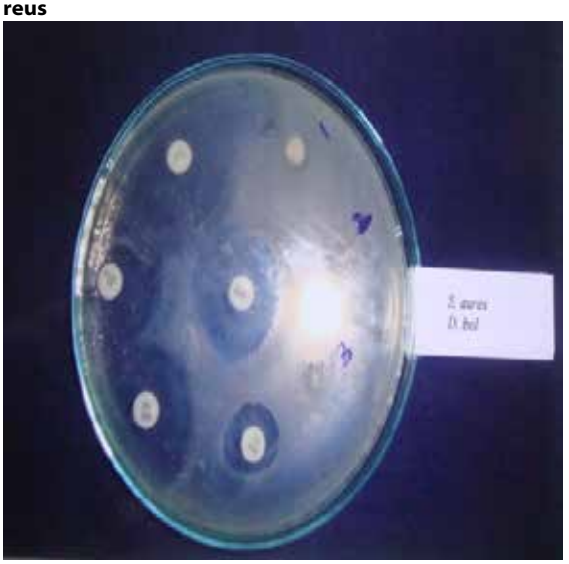
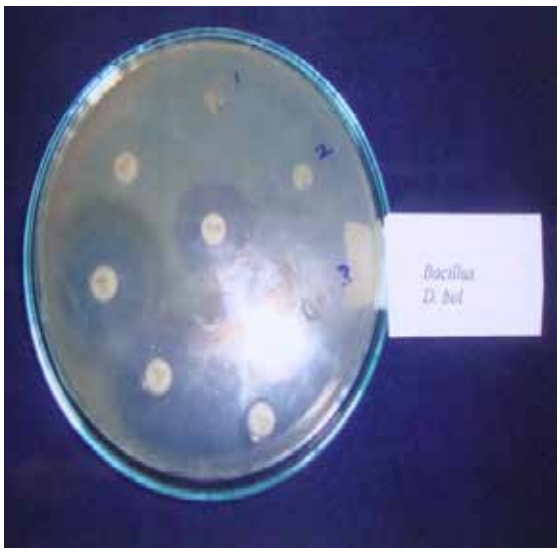
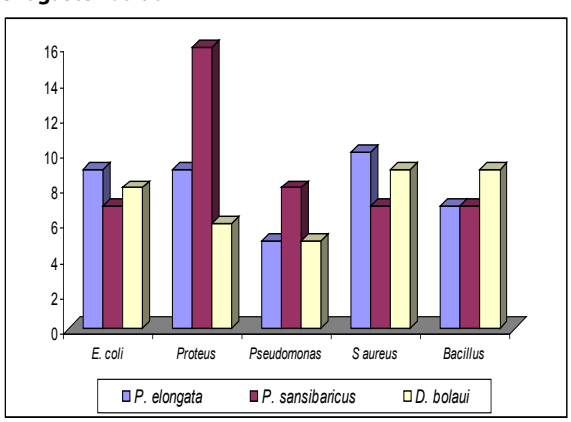


Plate – 5.15: Zone of suppression impregnated with coelomic fluid of Dichogaster bolaui on Bacillus



Graph 5.1: Showing inhibitory activity on pathogenic bacteria like Escherichia coli, Staphylococcus aureus, Pseudomonas, Proteus and Bacillus by Coelomic fluid of Polypheretima elongata, Perionyx sansibaricus and Dichogaster bolaui



5.4 DISCUSSION:

Preparations made out of materials of animal origin (placenta, snake and bee poison, dags, sea and river hydrocoels) were used extensively in folk and modern medicine for treating many human diseases. These preparations are also gaining importance now-a-days, though some of them are not used in modern medicine because of resource deterioration. Renewable natural medical materials of animal origin and pharmaceutical preparations on their basis as well as biologically active food supplements are the future. This area of human activity and economics should be developed and improved in every possible way. In many South-East Asia countries earthworms have already been used for nearly 2300 years to cure different human diseases. In Vietnam a dry earthworm powder produced according to different techniques that guarantee its purity is the compulsory ingredient of pharmaceuticals or "magic medicines that save life with in 60 minutes". These pharmaceuticals are often used to treat various organ dysfunctions-the result of bacterial and viral infections.

Darwin too commented that tissue fluid of earthworm can dissolve fibrin, many Japanese researchers have extracted fibrin dissolving enzymes from Lumbricus rubellus.

Our results show that coelomic fluid of earthworm *Polypheretima elongata*, *Perionyx sansibaricus* and *Dichogaster bolaui* applied in the certain concentrations could decrease growth of pathogenic bacteria.

In clinical experience, the inflection of skin wounds often occurs by Staphylococci causing a decrease in the proliferation of epithelial cells and fibroblasts in the wounds (Chang *et al.,* 1997). Maja *et al.* (2005) have reported G-90; a glycolipoproteins mixture obtained from tissues homogenate of earthworm *Eisenia fetida* and has shown the antibacterial activity *in vivo* and *in vitro* in different concentrations of growth on non pathogenic and facultative pathogenic bacteria. The bacteriostatic effect of the G-90 was 21% stronger for facultative pathogenic bacteria than that observed for non-pathogenic bacteria.

Upon the inoculation of bacteria into the coelomic cavity of earthworm, the coelomocytes initiate the process of connecting with each other by their adhesive structures around the bacteria and form so called brown bodies (Valembois *et al.,* 1992; Cooper *et al.,* 1999). At the same time the coelomocytes intensively synthesize and secrete proteins that adhere to the bacteria, forming aggregations and may inhibit their further proliferation. One of these proteins is agglutination of 56 kDa molecular mass, which attaches to the lectin like monosaccharide of the cellular membrane of the bacteria.

Rejnek (1991), Tuckova (1991) and Valembois *et al.* (1993) demonstrated significant antibacterial activity of the earthworms and that they were assisted besides coelomocytes by the chloragocytes, i.e., cell from the intestinal tract of earthworms, in resistance mechanisms. The chloragocytes secrete two proteins with a molecular mass of 40 and 45 kDa, which share 35% similarities with immunoglobulins. They adhere to the bacteria making them suitable for phagocytosis by coelomocytes in coelomic fluid (Lassegues *et al.,* 1997). The chloragocytes synthesis and secrete the protein lysenin (33 kDa), which binds specially to phospholipids of the cell membrane and causes cytolysis (Kobayashi *et al.,* 2000; Ohit *et al.,* 2000).

With the knowledge, it is difficult to define which molecule of coelomic fluid of earthworms is responsible for its antibacterial activity. Theoretically such activities could be ascribed to some of the following molecular masses: 33, 40, 42, 45 and 60 kDa, which are detected by SDS-PAGE in G-90 (Hrzenjak *et al.,* 1992). By immunochemical analyses these proteins are shown to be belonging to the immunoglobulin super family (Popovic *et al.,* 1998).

Looking at our results antibacterial activity of different species of earthworm is more in some species and less in some other species. This indicates that these earthworms show good antibacterial activity in particular species.

From the results of our study it is clear that antibacterial activity is exhibited at different levels by coelomic fluids of Polypheretima elongata, Perionyx sansibaricus and Dichogaster bolaui. Coelomic fluid of Polypheretima elongata is exhibit highest antibacterial activity against Escherichia coli and Staphylococcus aureus.

Perionyx sansibaricus has the highest antibacterial activity against Proteus and Pseudomonas; that of Dichogaster bolaui is against Staphylococcus aureus.

These observations are made based on zone of inhibition in vitro. However, further investigations on the presence of antibacterial molecules in the coelomic fluid and/or coelomocytes are needed which may help to explore the medicinal value of coelomic fluids of local species of earthworms.

5.5 SUMMARY AND CONCLUSION

1) Earthworms are known for their medicinal value since ancient times all over the world.
2) Earthworms have blood and the coelomic fluid that contains both haemocytes and coelomocytes in their fluids.
3) Coelomic fluids as well as blood of the earthworm have coelomocytes and haemocytes which play an important role in fighting against pathogens. This quality of earthworms made us to find out the immunological and antibacterial activity.
4) Our first part of the study was to know the types of coelomocytes present in the coelomic fluid of the earthworms. And in the second part of the study was to know the antibacterial activity of the coelomic fluid of the earthworms against pathogenic bacteria.
5) In our cellular study we found out mainly three types of coelo-

mocytes namely eleocytes, granulocytes and amoebocytes. In granulocytes and amoebocytes two sub types were found they are granulocytes I (G I), granulocytes II (G II) and in amoebocytes I (A I) and amoebocytes II (A II) types.

6) Second part of the study was know the antibacterial activity coelomic fluid of local species of earthworms like Polypheretima elongata, Perionyx sansibaricus and Dichogaster bolaui against pathogenic bacteria like E. coli, Proteus, Pseudomonas, S. aureus and Bacillus.
7) Results reveal that crude form of coelomic fluids of all the species of earthworm exhibited antibacterial activity. Polypheretima elongata and Dichogaster bolaui exhibit highest antibacterial activity against E. coli and S. aureus. Coelomic fluid of Perionyx sansibaricus has shown antibacterial activity against Proteus and Pseudomonas. These results are based on the zone of inhibition in vitro growth of bacterial cultures.
8) However, further investigations on the presence of antibacterial molecules in coelomic fluid of earthworms are necessary.

REFERENCES

1. Beschin, A., Bilej, M., Hanssens, F., Raymakers, J., Vandyck, E., Revets, H., Brys, L., Gomez, J., Debaetselier, P. and Timmermans, M., 1998. Identification and cloning of a glucan and lipopolysaccharide binding protein from Eisenia foetida earthworm involved in the activation of prophenoloxidase cascade. J. Biol. Chem., 237: 24948-24954. | 2. Cooper, E. L., Beschin, A. and Bilej, M., 2002. A new model for analyzing antimicrobial peptides with biomedical applications. Ist ed., NATO Science, New York, pp. 343-348. | 3. Cooper, E. L., Cossarizza, A., Kauschke, E. and Franceschi, C., 1999. Cell adhesion and the immune system: A case study using earthworm. Microse. Res. Tech., 44: 237-253. | 4. Cooper, E. L., Kauschke, E., Cossarizza, A., 2001. Annelid humoral immunity: Cell lysis in earthworms, Adv. Exp. Med. Biol., 484: 169-183. | 5. Hrzenjak, T., Hrzenjak, M., Kasuba, V., Efenbergez-Marinculic, P. and Levanat, S., 1992. A new source of biologically active compounds earthworms tissue (Eisenia foetida, Lumbicus rubellus). Comp. Biochem. Physiol., 102A(3): 441-447. | 6. Imegwu, O., Chang, T. H., Steinberg, J. J. And Levenson, S. M., 1997. Staphylococcus aureus peptidoglycan amliorates cyclophosphamide induced impairment of wound healing. Wound Rep. Reg., 5: 364-372. | 7. Jolles, P. and Zuili, S., 1960. Purification et etude compare de nouveaux lysozymes extraits du poumon de poule et de Nephtys hombergi, Biochem. Biophys. Acta, 39: 212-217. | 8. Kobayashi, H., Sekizawa, Y., Aizu, M. and Umeda, M., 2000. Lethal and non lethal responses of spermatozoa from a wide variety of vertebrates and invertebrates to lysenin, a protein from the coelomic fluid of the earthworm Eisenia foetida. J. Exp. Zool., 286: 538-549. | 9. Lange, S., Kauschke, E., Mohrig, W. and Cooper, E. L., 1999. Biochemical characteristics of Eiseoiapore, a pore forming protein in the coelomic fluid of earthworms. Eur. J. Biochem., 262: 547-556. | 10. Lassegues, M., 1986. Etude des activities antibacteriennes humorales et cellularies du lombricien Eisenia fetida andrei. These doctroates – Sciences Universite de Bordeauxi. | 11. Lassegues, M., Milochau, A., Doingnon, F., Du Pasquier, L. and Valembois, P., 1997. Sequence and expression of an Eisenia foetida derived cDNA clone that encodes the 40-kDa fetidin antibacterial protein. Eur. J. Biochem., 246: 756-762. | 12. Maja Popuic, Mira Grdisa and Terezija Mihaela Hrzenjak, 2005. Glycoliopo protein G-90 obtained from the earthworm Eisenia foetida exerts antibacterial activity. Verterinrska Arhiv., 75(2):119-128. | 13. Medzhitov, R. and Janeway, C. A., 1997. Innate immunity; the virtues of a non clonal system of recognition. Cell, 91: 295-298. | 14. Meszaros, G., Menesi, L. and Kopcsanyi, Z., 1993. Treatment of thermally injured patients with Betadine R. Solution and Cream. Therap. Hung, 4: 132-136. | 15. Ohita, N., Shioda, S., Sekizawa, Y., Nakai, Y. and Kobayashi, H., 2000. Sites of expression of mRNA for lysenin, a protein isolated from the coelomic fluid of the earthworm Eisenia foetida. Cell Tissues Res., 302: 263-270. | 16. Popovic, M., Hrzenjak, T., Grdisa, M. and Vukovic, S., 1998. Adhesins of immunoglobulin like super family from earthworm Eisenia foetida. Gen. Pharmacol., 30: 795-800. | 17. Rejnek, J., Tuckova, J., Zikan, M. and Tomana, 1991. The interaction of a protein from the coelomic fluid of earthworms with Staphylococcal protein A. Develop. Comp. Immunol, 15: 269-277. | 18. Scherbert, I. and Messner, B., 1997. Untersuchugen uber das vor kommenvon lysozyme bei anneliden, Zool. Jahrb. Physiol. Biol., 76: 36-50. | 19. Tuckova, L., Rejnek, J., Bilej, M. and Pospisil, R., 1991. Characterization of antigen binding protein in earthworms Lumbricus terrestris and Eisenia foettida. Develop. Comp. Immunol., 15: 263-268. | 20. Valembois, P., Lassegues, M. and Roch, P., 1992. Formation of brown bodies in the coelomic cavity of the earthworm Eisenia foetida Andrei and attendant changes in shape and adhesive capacity of constitutive cells. Develop Comp. Immunal., 16: 95-101. | 21. Valembois, P., Lassegues, M., Hirigoyenberry, F. and Seymour, J., 1993. Clearance and breakdown of pathogenic bacteria injected into the body cavity of the earthworm Eisenia foetida Andrei. Comp. Biochem. Physiol., 106C: 255-260. | 22. Valembois, P., Roch, P., Lassegues, M. and Cassand, P., 1982. Antibacterial activity of the hemolytic system from the earthworm Eisenia fetida Andrei. J. Invertebr. Pathol., 40: 21-27. |