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Full Length Research Paper

Antimicrobial activity of earthworm (Eudrilus *eugeniae)* paste

K. Vasanthi[1], K. Chairman[2] and A. J. A. Ranjit Singh[2]*

[1]Department of Zoology, Sri Parasakthi College for Women, Courtallam, M. S. University, Tirunelveli, Tamilnadu- 627 802, India.

[2]Department of Zoology, Sri Paramakalyani College, Alwarkurichi, M. S. University, Tirunelveli, Tamilnadu- 627 412, India.

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Earthworm plays a major role in the proper functioning of the soil ecosystem. It acts as scavenger and helps in recycling of dead and decayed plant material by feeding on them. Earthworm increases the soil fertility and is often referred to as a farmer's friend. Earthworms have been used in medicine for various remedies. The paste prepared from earthworm, *Eudrilus eugeniae* was tested for antibacterial, antifungal activities. For the antimicrobial screening, four species of bacterial isolate and two species of fungal isolates were selected. The bacterial cultures were used for antimicrobial testing maintained on nutrient agar slant and the fungal strains were maintained on Sabouraud dextrose agar slant at 4°C. Minimum inhibitory concentration (MIC) was determined using micro dilution broth method. Earthworm paste at a dose of 100 µl was able to inhibit the growth of bacteria of *S. aureus* at a maximum level as compared to other bacteria; the growth of fungal *Candida albicans* was much inhibited. The MIC results indicated that earthworm paste at a dose of 200 µl inhibited the bacterial growth. These studies may lead to the formulation of new antimicrobial drug. The antimicrobial activity of the paste was determined by an agar diffusion method using well and disc, the study clearly indicates that the paste contain a good antibacterial potential and the bioactive compounds to inhibit the growth of bacteria and fungi. Hence earthworm paste (EP) has a good potential to develop a new drug.

Key words: *Eudrilus eugeniae,* antimicrobial activity, fungal and bacteria strains.

INTRODUCTION

Earthworms have been used in medicine for various remedies since 1340 AD (Hossam et al., 2012). Earthworm has been recognized in oriental medicine as anti-inflammatory, analgesic and antipyretic agent (Prakash and Gunasekaran, 2010). It shows anticancer effect by preventing excess glucose uptake (Balamurugan et al., 2009). Microorganisms are known to play a major role in soil characteristics, invertebrates are believed to act as regulators of antimicrobial activity. Earthworms live in an environment filled with various kinds of pathogens. Physiologically and evolutionally speaking, earthworm survival in such an environment must have favoured the development of efficient defense mechanisms against various environmental pathogens during the course of evolution, including the production of certain anti-microbiological substances, especially active proteins and enzymes (Wenli et al., 2011). Earthworm surface excreta were found to have potent antimicrobial activity. It is also having anti-coagulatory or fibrinolytic activity which results in the facilitation of blood circulation (Cooper and Balamurugan, 2010). The earthworm has been suspected to contain proteases which dissolve the fibrin

*Corresponding author. E-mail: singhspkcc@gmail.com

Table 1. Antibacterial activity of crude earthworm paste in well assay method.

S/N	Organism	Zone of inhibition in concentration (mm) 50 µl	100 µl	Tetracycline (30 µl)
1	*Escherichia coli* (NCIM-2065)	16.33±0.33	22.66±0.33	24.12±0.26
2	*Salmonella abony* (NCIM-2257)	15.33±0.33	19.66±0.33	21.66±0.23
3	*Bacillus subtilus* (NCIM-2063)	17.66±0.33	20.33±0.33	23.42±0.17
4	*Staphylococcus aureus* (NCIM-2079)	10.33±0.33	15.33±0.33	18.43±1.21
5	*Klebsiella pneumonia* (Nosocomial isolate)	11.33±0.33	23.66±0.33	25.26±0.26

P-value = 1.22E-24.

Table 2. Antibacterial activity of crude earthworm paste in disc assay method.

S/N	Organism	Zone of inhibition in concentration (mm) 50 µl	100 µl	Tetracycline (30 µl)
1	*Escherichia coli* (NCIM-2065)	11.33±0.33	13.66±0.33	17.66±0.33
2	*Salmonella abony* (NCIM-2257)	11.33±0.66	11.33±0.66	17.66±0.33
3	*Bacillus subtilus* (NCIM-2063)	10.33±0.33	12.66±0.33	19.66±0.33
4	*Staphylococcus aureus* (NCIM-2079)	10.00±0.00	17.66±0.33	26.66±0.33
5	*Klebsiella pneumonia* (Nosocomial isolate)	10.33±0.33	13.66±0.33	17.33±0.33

P-value = 5.33E-13.

clots or anticoagulants which selectively interfere with the intrinsic pathway of blood coagulation cascade. Medicinal properties of earthworm had also been described (Shobha and Kale, 2007). Antimicrobial potency of *Eudrilus eugeniae* extracts on certain plant pathogens were studied (Bauer et al., 1966). Hence, in the present study, the paste prepared from the earth worm, *E. eugeniae* was tested for antimicrobial potential.

MATERIALS AND METHODS

Collection of earthworms

Fully matured Earthworms, *E. eugeniae* (Kinberg) were collected from the stock culture, Department of Zoology, Sri Paramakalyani College, Alwarkurichi, Tirunelveli, Tamilnadu, India.

Preparation of earthworm paste

The *E. eugeniae* (Kinberg) were washed with running tap water and then fed with wet blotting paper for 18 to 20 h to clear their gut. The gut cleared worms were again washed with distilled water. The worms were kept in plastic troughs, covered tightly with polythene cover, and exposed to sunlight for 3 days to kill them. Mucus and coelomic fluid that oozed out digested the dead worms forming a brown coloured paste earthworm paste (EP) (Balamurugan et al., 2007).The earthworm paste were filtered and the filtrates obtained were condensed in water-bath at 35°C. The crude paste obtained was diluted in 10% DMSO for evaluation of antimicrobial activity.

Culture media

The media used for bacterial culture was nutrient agar/broth while Sabouraud's Dextrose-Agar (SDA) was used for fungal cultures.

Microbial assay (disc and well method)

The antimicrobial activity of the earthworm paste was determined by an agar-diffusion method using well and discs. The details of microbes used for the testing is given in Table 1 and Plates 2 and 3.

Test microorganisms for antimicrobial studies

For the antimicrobial screening, four species of bacterial isolate and two species of fungal isolates were selected. The bacterial and fungal strains were obtained from National Collection of Industrial Microorganism (NCIM), Pune, India. *Escherichia coli* (NCIM 2065) *Salmonella abony* (NCIM 2257), *Bacillus subtilis* (NCIM 2063) and *Staphylococcus aureus* (NCIM 2079) strains were used. *Candida albicans* (NCIM 3102), *Aspergillus niger* (NCIM 501) fungi were used as test organisms. The nosocomial isolate and local isolate strains were received from Department of Microbiology, Aasan Memorial College, Chennai-100.

The bacterial cultures were used for antimicrobial test maintained on nutrient agar slant and the fungal strains were maintained on Sabouraud dextrose agar slant at 4°C. The fresh bacterial cultures were obtained by growing the test organisms at 37°C, for 24 h while fungi were grown at 28°C for 48 h.

Antibacterial assay

Antibacterial activity was determined against certified strains of *E. coli* (NCIM 2065) *S. abony* (NCIM 2257), *B. subtilis* (NCIM 2063), *S. aureus* (NCIM 2079), *K. pneumoniae* (Nosocomial isolate), using a modification of agar diffusion assay method (Shobha and Kale, 2007) filter-paper disc of 6 mm diameter, and 6mm sterile metal borer were used (Table 2). Microorganisms were inoculated on

Table 3. Antifungal activity of crude earthworm paste in disc assay method.

S/N	Organism	Zone of inhibition in concentration (mm) 50 µl100µl
1	*Candida albicans* (NCIM-3102)	12.33±0.3316.33±0.33
2	*Aspergillus niger* (NCIM-501)	11.00±0.5713.33±0.33
3	*Aspergillus flavus* (Local isolate)	10.33±0.3315.00±0.57
4	*Penicillium notatum* (Local isolate)	10.66±0.3314.33±0.33
5	*Trichophytum rubrum* (Nosocomial isolate)	0.00±0.0012.33±0.33
6	Nystatin (30 µl)	14.33±0.3326.66±0.33

P-value = 1.97E-16.

nutrient broth (Himedia, Bombay) during 24 h at 37°C. The inoculate absorbance was established between 0.08 and 0.10 AU (equivalent to 0.5 McFarland 10_8cfu/ml) adding sterile nutrient broth, before incorporating bacteria (λ = 625 nm). Bacterial strains were seeded on Muller-Hinton agar. The sterile discs were impregnated in the seeded agar. In the well assay method, wells were made using the sterile metal borer (6 mm). The sterile disc and well was loaded with the crude earthworm paste (EP) at a concentration of 50 and 100 µl. A disc of tetracycline (Tet) (30 µg/disc/well) was used as a positive control. The plates were incubated at 37°C for 24 h. All the assays were carried out in triplicate. The diameter (mm) of the growth inhibition zone was measured with standard zone reader scale (Himedia, Bombay) and mean diameter was recorded (Tables 2 and 3).

Antifungal assay

Antifungal activity was carried against *C. albicans* (NCIM 3102), *A. niger* (NCIM 501) *Trichophyton rubrum* (Nosocomial isolate), *A. flavus* (Local isolate), *Penicillium notatum* (Local isolate). The microorganisms were inoculated on Sabouraud dextrose broth (Hi-media, Bombay) during 24 h at 25°C. The inoculate absorbance was established between 0.08 and 0.10 AU (equivalent to 0.5 McFarland 10_8 cfu/ml) adding sterile Sabouraud dextrose broth, before incorporating fungi (λ = 530 nm). Fungal strains were seeded agar Sabouraud dextrose agar with 4% glucose. The sterile discs were impregnated in the seeded agar. In the well assay method, wells were made using the sterile metal borer (6 mm). The sterile disc and well were loaded with the crude earthworm paste (EP) at a concentration of 50 and 100 µl. Nystatin (100CFU/disc/well) antifungal agent was used as positive control. The plates were incubated at 25°C for 48 h. All the assays were carried out in triplicate. The diameter (mm) of the growth inhibition zone was measured with standard zone reader scale (Himedia, Bombay) and mean diameter was recorded.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was determined using micro dilution broth method. The earthworm paste prepared (100 mg/ml) were diluted to four different concentration (50, 100, 150 and 200 µl/ml).To find out the MIC, three strains of bacteria were used *(E. coli, K. pneumoniae* and *S. aureus).* The bacterial strains were prepared in broth. The broth culture suspension of each bacterium (0.5 ml) with an optical density of MacFarland 0.5 x 10_7-10_8 CFU/ml was added to test tubes containing different concentrations of earthworm paste. To the control test tube, the earthworm paste was not added, only sterile distilled water was used. The inoculated test tubes were incubated at 37°C under aerobic conditions. After 24 h, the turbidity was evaluated. The MIC is the lowest concentration of EP that inhibited the growth of the organism completely for all the tests including control, and triplicates were maintained.

RESULTS

Earthworm paste prepared from *E. eugeniae* was tested for antibacterial and antifungal activities. For antimicrobial assay, five strains of bacteria viz. *E. coli, S. abony, B. subtilus, S. aureus* and *K. pneumoniae* were used for antibacterial assay, two concentration of the EP 50 and 100 µl were used. Of the five bacteria tested, the growth of bacteria was well inhibited by EP. The disc diffusion assay and well assay indicated a dose dependent effect of the EP to inhibit the growth of bacteria. EP at a dose of 100 µl was able to inhibit the growth of *S. aureus* at a maximum level (17.66 ± 0.33 mm), *S. abony* was less inhibited when compared with others (11.33 ± 0.66 mm). In the well assay, *K. pneumoniae* was highly inhibited (23.66 ± 0.33 mm).

The present study clearly indicates that EP contains a good antibacterial potency and the active compound in it has to be explained.

Anti fungal assay was also carried out using five different strains of fungi. The fungal strain was inhibited by EP in a dose dependent manner. The high dose of EP (100 µl) showed highest anti fungal potential. Of the five strains tested, the growth of *C. albicans* was much inhibited (16.33 ± 0.33 mm). The inhibitory potential of the EP was less in the case of *T. rubrum* (12.33 ± 0.33 mm).

To find out the minimum inhibitory concentration (MIC) of the earthworm paste, three bacterial strains were used *Viz., E. coli, K. pnemoniae* and *S. aureus.* The MIC results indicated that earthworm paste at a dose of 200 µl inhibited the bacterial growth (Table 4). The optical density (OD) was less. This indicated that EP at a dose of 200 µl is a minimum concentration to inhibit the growth of the selected bacteria.

The present study clearly indicates that the earthworm paste has bioactive compounds to inhibit the growth of

Table 4. Antibacterial activity (MIC) tested for the crude earthworm paste using optical density method.

S/N	Bacterial culture	Concentration of crude earthworm paste	OD value
1	*Escherichia coli* (NCIM-2065)	Control	19.66±0.33
		50 µl	15.00±0.57
		100 µl	14.00±0.57
		150 µl	13.00±0.57
		200 µl	10.66±0.66
2	*Klebsiella pneumoniae* (Nosocomial isolate)	Control	15.00±0.57
		50 µl	13.33±0.66
		100 µl	9.33±0.33
		150 µl	7.66±0.33
		200 µl	7.33±0.66
3	*Staphylococcus aureus* (NCIM-2079)	Control	16.33±0.33
		50 µl	10.66±0.88
		100 µl	9.66±0.88
		150 µl	8.66±0.66
		200 µl	8.33±0.33

P-value = 0.000886; TNTC - Too numerable to count.

bacteria and fungi. Hence, EP has a good potential to develop a new drug.

DISCUSSION

Khomyakov et al. (2007) Suggested that antimicrobial agents of earthworms digestive fluid are formed in the earthworm body but not by the soil microorganisms entering their digestive tract. They have observed that the digestive fluid of earthworm show the same antimicrobial activity after feeding on soil and sterile sand, and partial sterilization of the gut with streptomycin does not lower the antimicrobial activity. Mendez et al. (2003) observed that the guts of earthworms, *Onychochaeta borincana* in sterile soil contain the same microorganisms as the guts of individuals that have not been submitted to the cleansing treatment. Antimicrobial activity in the guts of earthworms derived from metabolites of symbiotic bacteria from the gut walls is possible.

The antimicrobial activity of *Eisenia foetida* coelomic fluid directed against Gram-positive and negative bacteria was analyzed. The gut extracts of earthworms have antibacterial and antifungal activity (Shobha and Kale, 2008). The new bacterial strain with antimycobacterial activity has been isolated from the midgut of *Dendrobaena veneta* (Annelida) (Marta et al., 2010). Earthworm species have rich diversity. Based on their living environments, it is rational to think that there are effective anti-infective 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 (Cho et al., 1998). Recently, another two antimicrobial peptides (PP1 and OEP3121) have been identified from earthworms of *Pheretima tschiliensis* and *E. foetida,* respectively (Wang et al., 2003). The antimicrobial peptide, lumbricin-PG was identified from skin secretions of the earthworm, *Pheretima guillelmi* (Wenliang et al., 2011). 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.* Presumably, earthworms synthesize and secrete several effective modulators of innate immune responses such as antibacterial molecules, cytotoxic proteins and cytokines.

Conclusion

The burrowing and casting activities of earthworms contribute to the activity of soil micro organisms and nutrient enriched earthworm casts are good media supporting microbial growth. The microbial community in the gut of earthworms was predominantly Gram-negative bacteria. The present study reported that vermicompost is rich in microbial populations and diversity, particularly

fungi and bacteria. It is possible that earthworms can be used not only in environmental monitoring, but also in the acquisition of novel molecules for human therapeutic purposes. This study may thus lead to formulation of new natural antimicrobial agent and thus may be found to be beneficial in future prospects for mankind.

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