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SCREENING OF BACTERIA ISOLATES FROM EARTHWORM CAST FOR ANTIBACTERIAL ACTIVITIES

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ABSTRACT

The healthy operation of the soil ecosystem is significantly influenced by earthworms. Various medical treatments have included the use of earthworms. This study isolated and screened bacterial species from earthworm cast for antibacterial activities. Two (2) bacteria species were isolated from the earthworm cast by culturing on starch casein agar using pour plate techniques. The isolates were identified as Streptomyces and Actinomadura species while the test isolates include Klebsiella specie, Salmonella shigella and Staphylococcus aureus strains. This study revealed that Streptococcus and Actinomadura metabolite exhibited variable degrees of antimicrobial activities against the test isolates. The highest in-vitro antimicrobial activity is (12.0mm) was exhibited by the Streptococcus metabolite at the highest concentration of 100mg/ml against Staphylococcus aureus, while the least antimicrobial activity (8.0mm) was exhibited at the concentration of 80mg/ml and 40mg/ml against Salmonella species and Staphylococcus aureus. Actinomadura metabolite was effective against Staphylococcus aureus at the highest concentration of 100mg/ml. However there was significant difference observed in antibacterial activity of the metabolites when compared to the standard antibiotic (Gentamicin) ($p<0.05$). From the minimum inhibitory concentration (MIC) test result, Streptococcus metabolite demonstrated greater activity on Salmonella specie at the range of 3.12mg/ml and a lower activity on Staphylococcus aureus and Klebsiella specie at the range of 12.5mg/ml each. The Actinomadura metabolite demonstrated greater activity on Salmonella species at the range of 1.56mg/ml. Based on the findings of this research, earthworm (Pheretima posthuma) is a good source of antibacterial agents that can be identified and extracted as source of cheap medicines to control serious infections.

Keywords: Antibacterial; Antibiotics; Bacteria; Cast; Earthworm.

INTRODUCTION

Since 1340 AD, earthworms have been utilized in medicine for a variety of treatments (Hossam *et al*., 2012). According to Prakash and Gunasekaran (2010), earthworm is a known anti-inflammatory, analgesic, and antipyretic substance in oriental medicine. It exhibits anticancer effects via limiting the intake of extra glucose. Invertebrates are thought to function as regulators of antimicrobial action, while microorganisms are known to have a significant impact in soil

properties. Pathogens of many different types are present in the environment where earthworms dwell. According to biology and evolution, earthworm survival in such a setting must have favored the evolution of effective defense mechanisms against various environmental pathogens, including the production of specific anti-microbiological substances, particularly active proteins and enzymes (Wenli *et al*., 2011). The earthworm has been suspected to contain proteases that dissolve fibrin clots or anticoagulants that selectively interfere with the intrinsic pathway of blood coagulation cascade. Earthworm surface excreta were found to have potent antimicrobial activity. It also has anti-coagulatory or fibrinolytic activity, which results in the facilitation of blood circulation (Cooper and Balamurugan, 2010).

Traditional Chinese medicine (China), Kampo (Japan), and traditional Korean medicine all employ earthworms, including Pheretima, as a form of treatment (Cooper and Hirabayashi 2013). An indigenous earthworm found in Southeast Asia, East India, and Japan is called *Pheretima* sp. (Phylum: Annelida; Class: Clitellata; Order: Oligochaetales; Family: Megascolecidae) (Aspe and James 2014). According to research conducted on the *Pheretima* sp. in Japan, this species has a cylindrical body with many setae in each segment (Darmawan *et al*., 2012).

Earthworms' medicinal properties have been reported in numerous traditional Chinese medicine literature. However, little or no research addresses the antibacterial activity of the composition produced by earthworm endosymbiont. Most of the research on earthworm antibacterial activity uses direct earthworm extracts, such as Dharmawati *et al*. (2019), who extracted the antibacterial activity from the earthworm extract of *Lumbricus rubellus*, Chauhan *et al*. (2014). They examined the antimicrobial activity of the earthworm extract *Eudrilus eugeniae*. Earthworms are specifically used to treat seizures, febrile illnesses, or epilepsy (Shen, 2013). According to Brito-Vega and Espinosa-Victoria (2009), endogenous earthworms can also stimulate or inhibit important bacteria growth. The antibacterial composition produced by earthworms could result from the excretion of symbiotic bacteria that live in the intestines of earthworms.

Rarely have the antibacterial and industrial enzyme properties of earthworm castings been investigated. The casting action resulted in nutrition and microbial enrichment, according to a thorough literature review (Sharon and Paul, 2011). Therefore, compared to soil without casting activity, the quantity of total bacteria, siderophore-producing bacteria, and fluorescent *Pseudomonads* was higher in casts (Devliegher and Verstraete, 2018), however actinomycetes were not mentioned. Doube *et al*. (2014) investigated the relationship between earthworms, healthy soil microorganisms, and root pathogens and discovered that earthworms serve as carriers of healthy soil bacteria. As a result, there is a huge chance that new bacterial species will be found in earthworm casts together with brand-new bioactive substances. Therefore this study is aimed at isolating and screening bacterial species from earthworm cast for antibacterial activities.

MATERIALS AND METHODS

Collection of Samples

In August 2022, during the rainy season, twelve (12) earthworm (*Pheretima posthuma*) castings were gathered from various locations in Umuariaga village, Ikwuano, Umuahia, Abia state, Nigeria. Each location yielded four samples. With the use of a spatula, the earthworm castings were carefully removed

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and stored in sterile polypropylene bags. The collected earthworm castings were brought to the laboratory for bacterial isolation, identification, and screening for antibiotic potential.

Preparation of Culture Media

The media used were Mannitol Salt Agar, MacConkey Agar, Salmonella and shigella Agar, and peptone water. They were prepared according to the manufacturer's instruction of each medium. The required amount of the powdered medium was weighed following manufacturer's specification and dissolved in distilled water in a conical flask. The dissolved media were autoclaved at 121°C for 15 minutes.

Isolation of Bioactive Bacteria from the Earthworm Cast Samples

Pre-Treatment of the Earthworm Cast

The earthworm cast (puddle soil emitted from the earthworm's gut) was first treated with phenol to reduce the growth of the non-bioactive *Streptomyces* species thereby facilitating the recovery of bioactive *Streptomyces* species that are known for their antibacterial and antitumor activities as described by Kamarudheen *et al*, (2014).

Phenol Treatment

One gram (1g) of the earthworm cast was dissolved in 9ml of sterile 0.85% of NaCl, then 1ml of the suspension was taken and added into 9ml of 1.5% phenol solution. It was incubated for 30 minutes at room temperature and serial dilutions were made up to 10^6 inoculations (Janaki *et al*., 2014).

Isolation

From the dilution 10^6, 0.1ml (100microliter) was taken and inoculated on starch casein agar, supplemented with nystatin and nalidixic acid to inhibit fungi and bacteria contamination respectively. The plates were then incubated for seven (7) days at 28°C.

Purification of Isolates

By sub-culturing on recently made Nutrient Agar plates, the colonies that emerged from the starch casein agar plates were purified. The plates were incubated for 24 hours at 35 °C. The resultant distinct colonies were kept in agar slant for further use after an overnight incubation period.

Identification of the Bacteria Isolates

The bacterial isolates were analyzed based on, Gram staining, morphological features and biochemical characterization which includes; coagulase, catalase, citrate, oxidase, motility, indole and urease tests of the isolates were carried out to verify the identity of the organisms. Using Bergey's manual of determinate bacteriology (2008), the bacterial isolates were identified and their identities confirmed.

Screening of Soil Isolates for Antibiotic Production

The capacity of certain of the bacterial isolates to produce antibiotics against the other test species was tested using a sensitivity assay. This method was used to illustrate Alexander Fleming's observation of the penicillin discovery.

Production of Antibacterial Metabolites from the Actinomycetes species

Each of the *Actinomycetes* species (*Streptomyces* and *Actinomadura*) were grown separately in starch casein broth, and incubated for 7days at 28°C. The broths were centrifuged and their supernatants were obtained separately and labeled.

Preparation of Paper Disc

This was prepared by using a Whatman filter paper which was cut in circle of 6mm with aid of an office paper perforator. The cut discs were boiled in distilled water for an hour (to remove possible residual preservatives), drained dry and sterilized by autoclaving.

Preparation of different concentration of the Metabolites

The metabolites were reconstituted by weighing 0.2 g quantity of each metabolite into a sterile test tube, and made up to 2 ml using distilled water to give a concentration of 200 mg/ml. This 200 mg/ml concentration of each metabolite was then, doubly diluted in sterile water to obtain concentration 100mg/ml, 80mg/ml, 40mg/ml, 20mg/ml, 10mg/ml and 5mg/ml.

Test Microorganisms for Antimicrobial Studies

For the antimicrobial screening, four species of bacterial isolates were selected. The bacterial strains were obtained from already made stock culture plate at O. J. BS laboratories, Umuahia, Abia State, Nigeria. *Klebsiella* species, *Salmonella shigella* and *Staphylococcus aureus* strains were used. The bacterial cultures were kept on nutrient agar slants at 4°C and utilized for antimicrobial tests. The test organisms were grown for 24 hours at 37°C to produce fresh bacterial cultures.

Determination of Antimicrobial Activity

The different bacteria obtained from the stock culture were then inoculated respectively into the solid sterile Muller Hinton agar. This was done using streak plate method. After the inoculation of the isolates, the prepared discs were dipped into the water concentration of the isolates from soil-cast and allowed to absorb it. Carefully, with the aid of a flame pair of forceps, the water concentrations of the isolates from soil-cast bearing discs were transferred to the inoculated plate. Three discs containing the inoculums were used for each plate and they were placed about the same distance from one another and not less than 1cm from the edge of the Petri dish. The plates containing the bacteria were incubated at 37^{O}C for 24hours and examined after three days of incubation. The plates containing bacteria growth were then examined the following day for zone of inhibition. Using a clear rule, the diameters of the inhibitory zone were measured and recorded in millimeters (mm). The test was run twice, and the average values were computed and noted. Any disc that had a clean zone surrounding it had good results.

Determination of Minimum Inhibitory Concentration (Mic) of the Metabolites Using Broth Dilution Technique

The Minimum inhibitory concentration (MIC) of the metabolites on the test isolates were determined by the broth dilution method. Metabolite concentration (100mg/ml) was further diluted in a 2-fold serial dilution to obtain the following concentrations: 50, 12.5, 6.25, 3.12, 1.56 and 0.78mg/ml. about 1ml stock solution of the metabolites 100mg/ml was diluted in sterile test tube containing 0.95ml of Mueller Hinton Broth (MHB) to obtain further dilution. Serial dilution techniques were employed by transferring 1ml from the first test tube to the second test tube and from the second to the third. This was continued to the seventh test tube from where 1ml was discarded to give concentration of 50, 12.5, 6.25, 3.12, 1.56 and 0.78mg/ml Another test tube was also prepared in a similar way containing MHB and inoculated with standard suspension (50µl) of the test organisms and incubated at 37°C overnight. This served as a control tube. After incubation, growth of the organisms in form of turbidity in each tube was checked. The minimum dilution

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(concentration) of the extracts completely inhibiting the growth of each organism was taken as the MIC for the organism tested.

Statistical Analysis

Data obtained was statistically analyzed. ANOVA on ranks was carried out and P value was determined.

RESULTS

Table 1 shows the bacterial species which were obtained from the earthworm cast and identified by morphological characteristics, pigmentation on media, microscopy, and biochemical methods. The bacterial isolates include *Streptomyces* species and *Actinomadura* species. Both isolates were then cultured for 24 hours and then purified. The gram staining results showed that *Streptomyces* species and *Actinomadura* species were categorized as the gram-positive group.

The antimicrobial activity of the earthworm cast isolate (*Streptococcus* metabolite) against the test bacteria. The *Streptococcus* metabolite exhibited variable degrees of antimicrobial activities against the test isolates. This was shown by the clear zones of inhibition produced by the metabolite on the test microorganisms. The highest in-vitro antimicrobial activity is (12.0mm) was exhibited by the *Streptococcus* metabolite at the highest concentration of 100mg/ml against *Staphylococcus aureus*, while the least antimicrobial activity (8.0mm) was exhibited at the concentration of 80mg/ml and 40mg/ml against *Salmonella* species and *Staphylococcus aureus* respectively. However at concentrations 20mg/ml, 10mg/ml and 5mg/ml, the test organisms were resistant to the *Streptococcus* metabolite (Table 2).

Table 3 shows the antimicrobial activity of the earthworm cast isolate (*Actinomadura* metabolite) against the test bacteria. The *Actinomadura* metabolite exhibited variable degrees of antimicrobial activities against the test isolates. This was shown by the clear zones of inhibition produced by the metabolite on the test microorganisms. The highest in-vitro antimicrobial activity (13.0mm) was exhibited by the *Actinomadura* metabolite at the highest concentration of 100mg/ml against *Staphylococcus aureus*, while the least antimicrobial activity (8.0mm) was exhibited at the concentration of 80mg/ml and 40mg/ml against *Salmonella* species and *Staphylococcus aureus* respectively. However at concentrations 20mg/ml, 10mg/ml and 5mg/ml, the test organisms were resistant to the *Actinomadura* metabolite.

The minimum inhibitory concentration (MIC) test result. The antibacterial activities of *Streptococcus* metabolite were investigated on *Staphylococcus aureus*, *Klebsiella* species and *Salmonella* species. The *Streptococcus* metabolite was active against all the test isolates but demonstrated greater antimicrobial activity on *Salmonella* species at the range of 3.12mg/ml and a lower activity on *Staphylococcus aureus* and *Klebsiella* species at the concentration range of 12.5mg/ml (Table 4).

Table 5 shows the minimum inhibitory concentration (MIC) test result. The antibacterial activities of *Actinomadura* metabolite were investigated on *Staphylococcus aureus*, *Klebsiella* species and *Salmonella* species. The *Actinomadura* metabolite was active against all the test isolates but demonstrated greater antimicrobial activity on *Salmonella* species at the range of 1.56mg/ml.

Table 1: Biochemical Identification of the Test Organisms

Colonial features	Gram Reaction	Cell Arrangement	Catalase	Oxidase	Coagulase	Indole	Citrate	Motility	Methyl Red	Voges-P	H_2S	Urease	Glucose	Lactose	Suspected bacteria
Creamy coloured colonies	+	Short Rods	+	-	-	+	-	-	-	-	-	-	+	+	*Streptomyces* species
White pink round colonies	+	Short Rod	+	-	-	+	-	-	+	-	-	-	+	-	*Actinomadura* species

Key: - = Negative + = Positive

Table 2 Antimicrobial activities of the earthworm cast isolate (*Streptococcus* metabolite) against the test bacteria

Test Organisms	Concentration (mg/ml)/Zone of Inhibition (mm)					
	100mg/ml	**80mg/ml**	**40mg/ml**	**20mg/ml**	**5mg/ml**	**GEN (C_T)**
Staphylococcus aureus	12.0	10.0	8.0	0.0	0.0	21.0
Klebsiella species	11.0	9.0	0.0	0.0	0.0	19.0
Salmonella species	10.0	8.0	0.0	0.0	0.0	19.0

Key: GEN (C_T) = Gentamicin (Control), milligram/mil (mg/ml), millimeters (mm)
P-value = 0.05

Table 3 Antimicrobial activities of the earthworm cast isolate (*Actinomadura* metabolite) against the test bacteria

Test Organisms	Concentration (mg/ml)/Zone of Inhibition (mm)					
	100mg/ml	**80mg/ml**	**40mg/ml**	**10mg/ml**	**5mg/ml**	**GEN (C_T)**
Staphylococcus aureus	13.0	12.0	8.0	0.0	0.0	21.0
Klebsiella species	11.0	9.0	0.0	0.0	0.0	19.0
Salmonella species	9.0	8.0	0.0	0.0	0.0	19.0

Key: GEN (C_T) = Gentamicin (Control), milligram/mil (mg/ml), millimeters (mm)
P-value = 0.05

Table 4: Minimum inhibitory concentration of earthworm cast isolate (*Streptococcus* metabolite) against the test bacteria

	Tests organisms		
Concentration (mg/ml)	***Staphylococcus aureus***	***Klebsiella* species**	***Salmonella* species**
50	-	-	-
25	-	-	-
12.5	-	-	-
6.25	+	+	-
3.12	+	+	-

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1.56	+	+	+
0.78	+	+	+
MIC Value (mg/ml)	**12.5**	**12.5**	**3.12**

Key: – no growth of the isolates in the tube shown by clarity in the tubes, + turbidity in the tubes indicating growth of the isolates, MIC = Minimum Inhibitory Concentration, milligram/mil (mg/ml).

Table 5: Minimum inhibitory concentration of earthworm cast isolate (*Actinomadura* metabolite) against the test bacteria

	Tests organisms		
Concentration (mg/ml)	***Staphylococcus aureus***	***Klebsiella* species**	***Salmonella* species**
50	-	-	-
25	-	-	-
12.5	-	-	-
6.25	-	-	-
3.12	+	+	-
1.56	+	+	-
0.78	+	+	+
MIC Value (mg/ml)	**6.25**	**6.25**	**1.56**

Key: – no growth of the isolates in the tube shown by clarity in the tubes, + turbidity in the tubes indicating growth of the isolates, MIC = Minimum Inhibitory Concentration, milligram/mil (mg/ml).

DISCUSSION

This study was to isolate and screen bacterial species from earthworm cast for antibacterial activities. According to Caulier *et al.* (2019), the capacity of diverse *Actinomadura* and *Streptomyces* species to create antibiotic chemicals supports their ability to thrive everywhere, including on land, in water, on food, and inside intestinal animals. Despite the fact that there are many good findings on the separation of bacteria from earthworm castings in agricultural soil, there are less data on the antibacterial activity of these bacteria (Ruanpanun and Chamswarng, 2015). Additionally, it can be a vital resource for finding novel or uncommon actinobacteria, which could produce valuable bioactive compounds.

Only the *Streptomyces* species from castings that were hostile to the typical litter and wood decomposer fungi were reported by Jayasinghe and Parkinson, (2009). There was no information provided on the antibacterial activity of the nine Streptosporangium strains from earthworm castings that Mba (1996) reported had phosphate solubilizing activity. Karsten and Drake (1997) who found number of microorganisms (bacteria, actinomycetes, fungi) in alimentary tract earthworms were six times higher in comparison with the surrounding soil. The antibacterial resistance is presently an urgent biological control of focus of research and new antibiotics are necessary to combat pathogens.

According to Khomyakov *et al*. (2007), soil microorganisms do not develop antimicrobial agents in the digestive fluid of earthworms;

rather, these agents are created within the body of the earthworm itself. They have shown that after consuming dirt and sterilized sand, earthworm digestive fluid exhibits the same antibacterial activity, and partial sterilization of the stomach with streptomycin has no effect on this antimicrobial activity. Mendez *et al*. (2003) discovered that *Onychochaeta borincana* earthworms in sterile soil had the same bacteria on their intestines as people who have not undergone the cleaning procedure. It's likely that metabolites of symbiotic bacteria from the gut walls have an antimicrobial effect in the intestines of earthworms.

Our research finding reported that *Streptomyces* metabolites showed actively antimicrobial activity against one or more tested human pathogens, *Staphylococcus aureus,* and *Klebsiella pneumoniae* as seen the clear zone inhibition (Table 2). As a result, *Staphylococcus aureus* MTCC2940, *Candida albicans* MTCC1637, *Microsporum canis* MTCC3070, and *Macrophomina phaseolina*, plant pathogens, were examined for antibacterial activity using the isolates from earthworm cast that were discovered, according to Kumar *et al*. (2012). As a consequence, early records of the *Streptomyces* spp. actively dominating species for antibacterial properties have been made, and further research is being done on their active metabolites and other responses. As opposed to other genera, *Streptomyces* accounts for over 80% of all antibiotic products, according to earlier research (Kieser *et al*., 2010).

From the present study, the zones of inhibition produced by *Actinomadura* metabolite ranged from 0.0mm at 5mg/ml to 13mm at 100mg/ml against *Staphylococcus aureus*; 0.0mm at 5mg/ml to 11.0mm at 100mg/ml against *Klebsiella* species; 0.0mm at 5mg/ml to 9.0mm at 100mg/ml against *Salmonella* species (Table 2). The most sensitive organisms to the *Actinomadura* metabolite were *Staphylococcus aureus* while *Salmonella* species was the least sensitive. According to recent research, earthworm extract has strong antibacterial efficacy. These findings are consistent with those of prior studies that found coelomic fluid had antibacterial action against bacteria (Bansal *et al*., 2015; 2016).

The minimum inhibitory concentration of *Streptococcus* and *Actinomadura* metabolite against test bacteria as revealed in this study indicates that the metabolites had activity against all the isolate but showed greater inhibitory activity against *Salmonella* species at the range of 3.12 mg/ml and 1.56 mg/ml respectively (Table 4 and Table 5). The finding by Prescott *et al*. (2008) that bacteria differed greatly in the degree of their sensitivity appeared to be consistent with the discovery reported regarding the minimum inhibitory concentration (MIC) of the metabolites.

The findings of the current study concur with those of Prakash and Gunasekaran (2011). They discovered that *S. aureus, P. mirabilis*, and *P. aeruginosa* were all very sensitive to the earthworm cast of two species (*Lampito maurtii* and *Perionyx excavatus*). The results of this study are further supported by Hatti (2014), who noted that *Polypheretima elongata*'s coelomic fluid had the strongest antibacterial effect against *Staphylococcus aureus*. According to Khomyakov *et al.* (2007), soil microorganisms do not develop antimicrobial agents in the digestive fluid of earthworms; rather, these agents are created within the body of the earthworm itself.

It is widely known that the formation and spread of antimicrobial resistance is a severe issue on a global scale (Gold and Moellering, 2016). Threatening to take us back to the time

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before the invention of antibiotics is the detection of novel emerging bacterial resistance (Smith *et al*., 2019). This situational production advises that new, safe, and efficient antimicrobials be researched in order to replace outdated antimicrobials (Gerding *et al*., 2011). Actinomycetes have been recognized as source of several secondary metabolites, antibiotics and lytic enzymes among which *Streptomyces* spp. have been shown to have characteristics which make them useful as antagonistic agents against pathogens.

CONCLUSION

Because antibiotics are used often and improperly in clinical studies, bacterial strains are becoming more and more resistant to antibiotics. There is an urgent need to create or find novel antibacterial agents in order to solve this issue. The goal of this study was to present a novel source of antibacterial agents as a powerful all-natural substitute for antibiotics. The antibacterial properties of several actinomycete isolates from soil samples were described in the current investigation. Based on the findings of this research, *Streptomyces* and *Actinomadura* metabolites demonstrated greater antimicrobial activity on *Salmonella* species. This indicates the presence of antimicrobial agents body paste. Thus, the antibacterial compounds found in earthworms (*Pheretima posthuma*) need to be discovered and extracted in order to be used as a source of low-cost medications to treat severe illnesses.

Recommendation

- Intensive investigation into different species of *Actinomyces* with metabolites can lead to commercialization of the product, this in turn can help boost employment, and increase raw materials for industries like pharmaceuticals.

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Conflict of Interest

Authors have declared that no conflict of interests exists.

Authors' Contribution

This work was carried out in collaboration among all authors. Author CGU designed the study, wrote the protocol, and performed the statistical analysis. IUN wrote the first draft of the manuscript and EKC helped with the analyses of the work. All authors read and approved the final manuscript.

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