Investigating the antibacterial, antioxidant and antifungal effects of *Lumbricus terrestris*

Group 1-08

Abstract:

This project investigates the antibacterial, antioxidant, and antifungal effects of *Lumbricus terrestris*, also known as earthworms. They live in a bacteria-abundant environment and have astonishing wound-healing capabilities. When half of its body is severed, the half with the head will grow back the other half over time. In doing so, it overcomes potential bacteria and fungal infections in its environment. Hence, we believe that *Lumbricus terrestris* have antibacterial and antifungal properties to aid in their wound-healing. It is also able to prevent oxidative damage to its cells to restore themselves. We carried out a well diffusion assay and a time point assay to test the earthworm extract for any antibacterial properties and carried out a DPPH (2,2-diphenyl-1-picrylhydrazyl) assay to test for antioxidant properties. Additionally, we conducted an antifungal assay to test for antifungal properties. We found out that the earthworm extract has strong antibacterial and antifungal properties but has weak antioxidant properties. We believe the active compounds of the earthworm extract can be used in creating an antiseptic solution to mitigate wound infections.

Introduction:

Chronic wounds are wounds that do not heal within 30 days(Chronic Wounds,n.d.), and are usually caused by high levels of oxidative stress(*Fitzmaurice SD, et al.,2012*). Since chronic wounds are unable to heal, they become the site of entry for harmful microbes, increasing one's susceptibility to wound infection. In extreme cases, when the infections are inadequately treated, the victim may have to remove a limb. In fact, chronic wounds infection affects 6.5 million people in US alone (Nelson, 2017), highlighting the severity of the problem.

Bacteria are gaining resistance to most antibiotics we must therefore find new ways of treating wound infections. As such, new medicine must be discovered to mitigate this problem. We believe that *Lumbricus terrestris* is a possible candidate for antibacterial, antifungal and antioxidant active compounds. These organisms live in an environment with a high microbial

load(Ingham,n.d.). They also have a fast wound healing rate(Castro, 2019), which may be assisted by antioxidants in its body. Research done on the earthworm species, *Eisenia foetida*, and *Lumbricus rubellus* proves that they have antioxidant properties as it was able to protect the cells from oxidation and allowed the cells to grow. This project aims to investigate the antibacterial, antifungal and antioxidant abilities of *Lumbricus terrestris* extract. This is a species commonly found in Singapore and would serve as an easily accessible local source for potential drug development.

Hypothesis:

We hypothesise that earthworm extract will display strong antibacterial, properties on both Gram-negative and Gram-positive bacteria. We also hypothesis that earthworm extract will display strong antifungal and antioxidant properties.

Methodology:

Extraction:

Lumbricus terrestris kept in soil was purchased from a local compost farmer. Afterwards, they were brought to the lab and washed until no visible debris could be seen. They were then put into a blender. Deionized water was added to the blender such that a 50% (m/v) extract would be obtained. Once blended, the mixture was centrifuged at 9500 rpm for 15 mins at 5˚C to remove large debris. The aqueous mixture was then decanted from the suspension. Afterwards, the mixture was filtered through a microfilter (pore size 0.45um) to remove all bacteria and ensure sterility in downstream tests. The supernatant was aliquoted and stored at -4˚C until required for further usage.

Well Diffusion Assay:

Bacteria were cultured in nutrient broth at 37 ˚C overnight. The resultant suspension will be used for subsequent testing. Using a sterile cotton swab, the bacteria were spread evenly onto agar plates. 3 wells were punched onto these inoculated agar plates. 100µL of either earthworm extract, 10% of bleach (positive control) and sterile water (negative control) were introduced into

each well. The set up were incubated at 37 ˚C overnight and the resultant bacterial growth were observed. Any resultant zones of inhibition generated were measured.

Time Point Assay:

Bacteria were cultured overnight in nutrient broth, quantified and its numbers adjusted to a starting concentration of 10^6 CFU/mL. The bacterial suspension was then mixed with either 100 µL of earthworm extract, 10% bleach (positive control), or sterile water (negative control). Samples of the mixtures were taken at 20-minute intervals, serially diluted and plated onto fresh agar plates. These agar plates were incubated at 37 ˚C overnight. The resultant colonies were counted and the actual bacterial numbers at various time points were calculated.

Antifungal Assay:

Fungus was cultured on potato dextrose agar for a few days at room temperature. Potato dextrose agar was then mixed with *Lumbricus terrestris* extract and was left to solidify. Once it solidified, a small layer of fungus were cut out with a sterile scalpel and placed in the middle of either the potato dextrose agar (negative control) or the agar with *Lumbricus terrestris* extract mixed with it. The resultant fungal surface growth were monitored for a week.

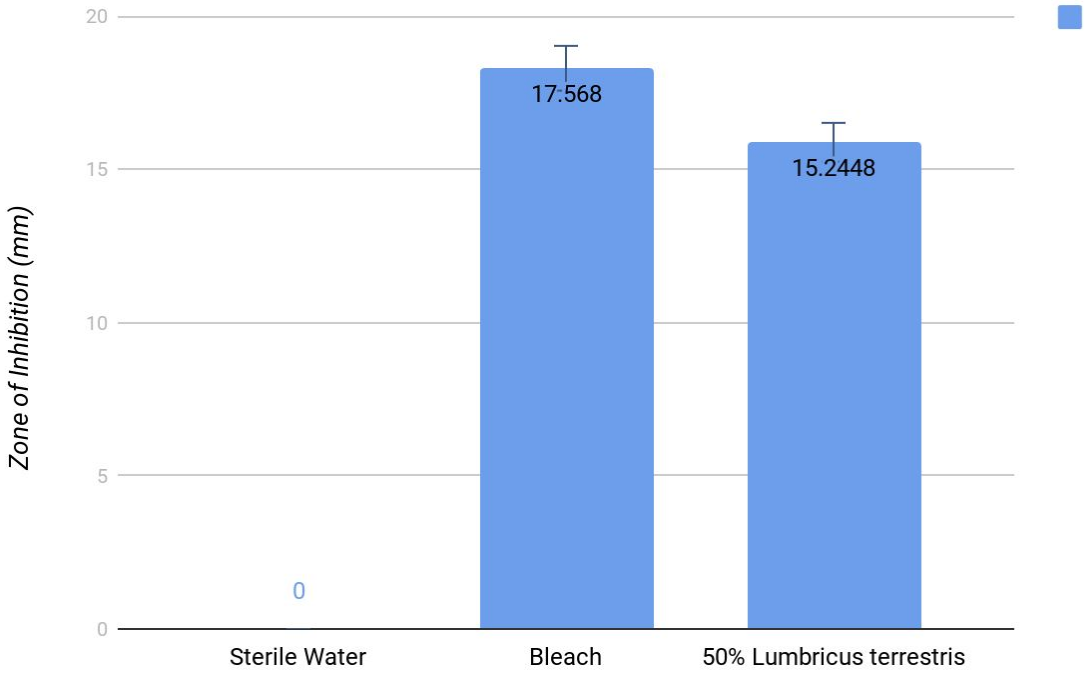
DPPH Assay:

1.9 mL of methanol was mixed with 0.1 mL of earthworm extract and 1 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. A negative control containing 2 mL of methanol and 1mL of DPPH solution were prepared. The tubes were placed in the dark for 10 minutes before spectrophotometric measurements were taken at 517 nm wavelength of light. The measurements were compared with those obtained from the negative control. By calculating the percentage of DPPH left, the antioxidant effects of the extract was determined.

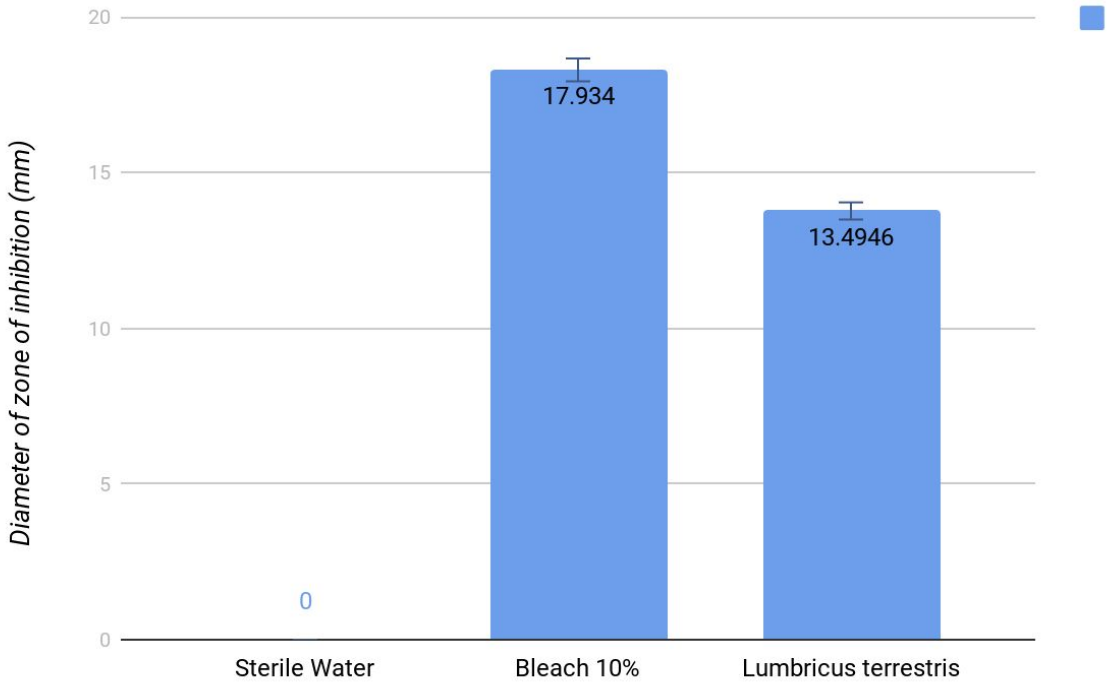
$$\left(\frac{A_{517}\text{ of control} - A_{517}\text{ of sample}}{A_{517}\text{ of control}}\right)\times 100$$

Results and Discussion:

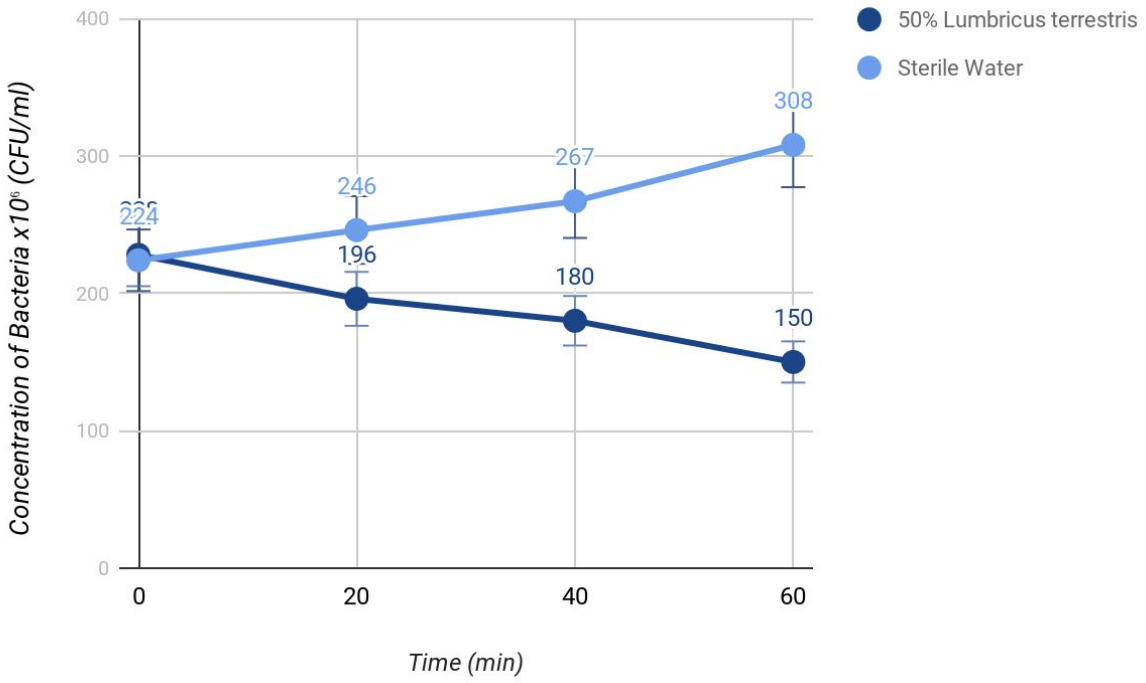
Graph 1: Average diameter of Zone of Inhibition (mm) generated by earthworm extract against *S. epidermidis*



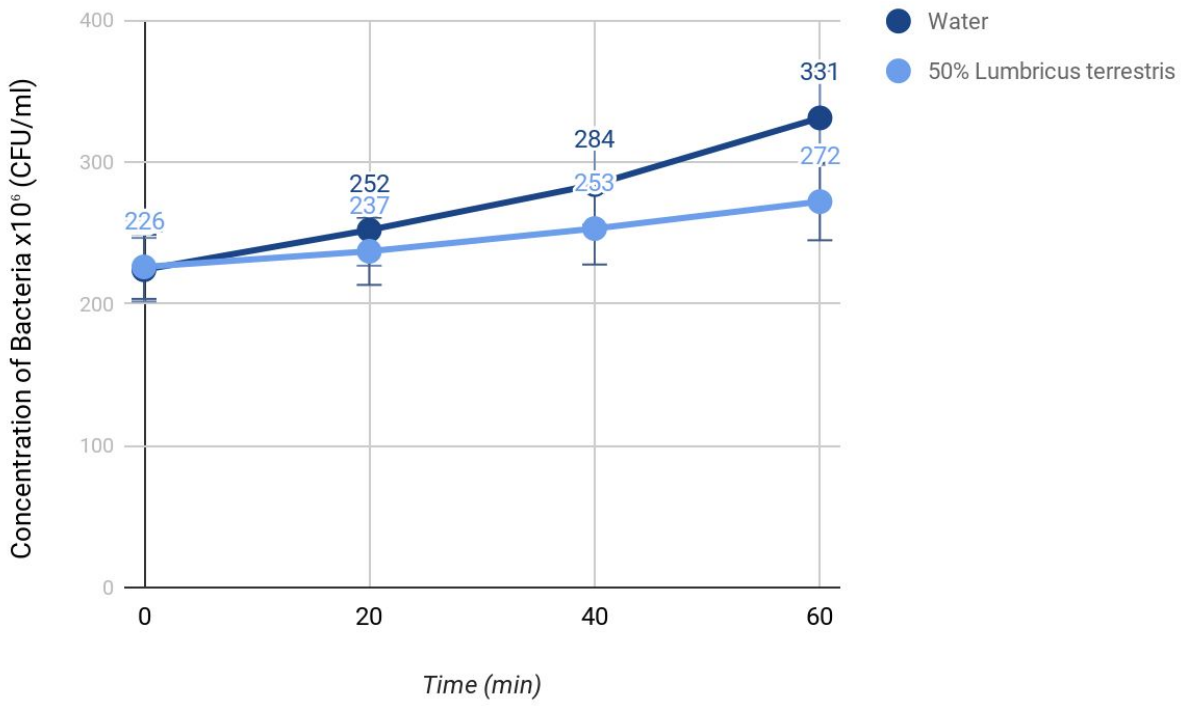
Graph 2: Average diameter of Zone of Inhibition (mm) generated by earthworm extract against *E.coli*



Graph 3: Effect of earthworm extract on the concentration of *S. epidermidis* ($\times 10^6$ CFU/ml) over time (min)

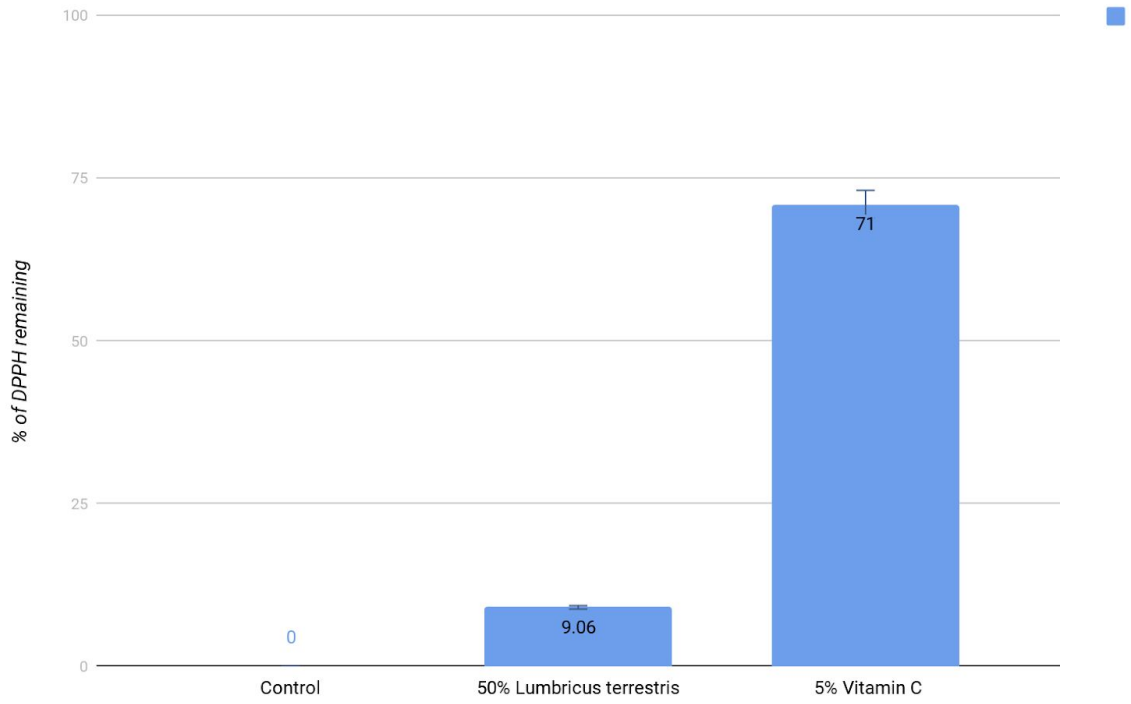


Graph 4: Effect of earthworm extract on the concentration of *E. coli* ($\times 10^{6}$ CFU/ml) over time (min)



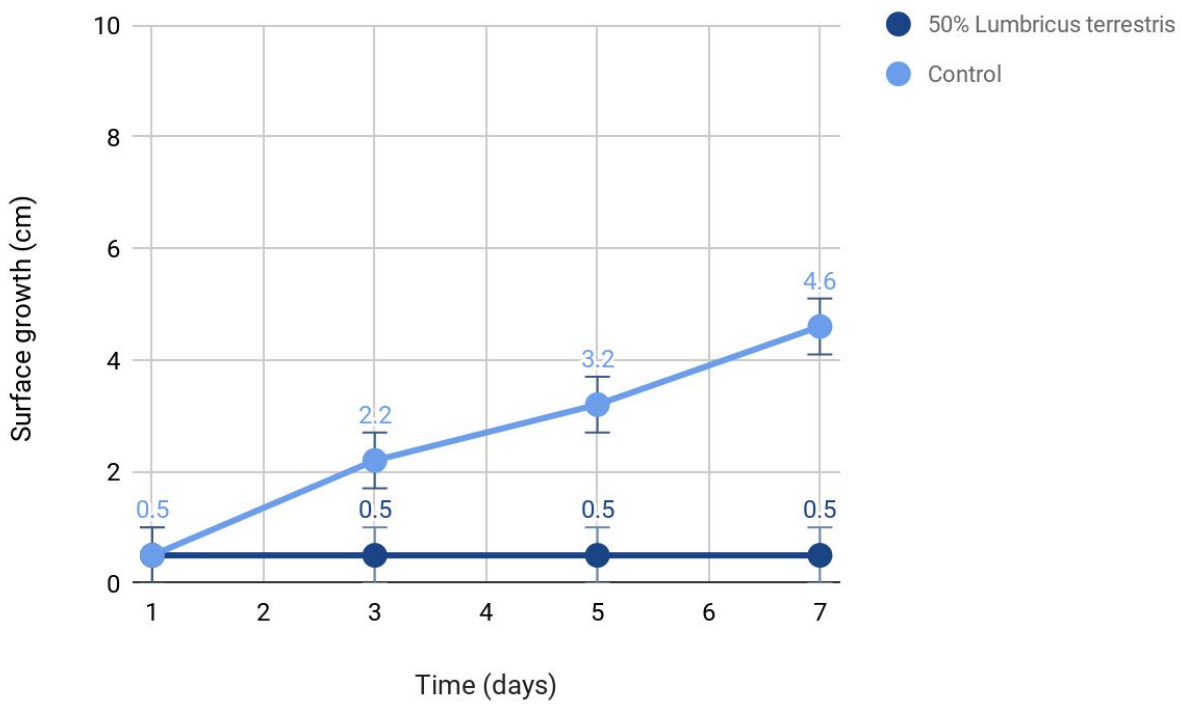
The bacteria count reduced by approximately 50% in the first hour of application of earthworm extract for *Staphylococcus epidermidis*, proving that it was bactericidal for Gram-positive bacteria. In contrast, the bacteria count was reduced by approximately 17.8% in the first hour of application of extract for *Escherichia coli.* This proves that the extract displayed bactericidal properties to a lesser extent for Gram-negative bacteria. However, the fact that it is able to display bactericidal effects on Gram-negative bacteria is valuable as the outer membrane in this type of bacteria protects it from many antibiotics currently in use, such as Penicillin.

Graph 5: DPPH Assay (% of DPPH remaining)



Earthworm extract was able to scavenge 9% of DPPH activity while a 5% vitamin C extract (positive control) was able to scavenge 71% of DPPH activity. This proves that earthworm extract is a weak antioxidant at best. However, earthworm extract was only able to scavenge 9% of DPPH. The fact that a commonly available antioxidant such as vitamin C could scavenge about 8 times of DPPH than earthworm extract shows that earthworm extract is a weak antioxidant. The results disproves our hypothesis that earthworm extract has antioxidant properties.

Graph 6: Effect of earthworm extract on the surface growth of *A. niger* (cm) over time (Days)



Earthworm extract is a strong antifungal agent as it reduced the fungal diameter growth by 88.8% in the first week of application. This proves that earthworm extract is a strong antifungal agent.

Limitations:

The solvent used to dilute the extract was water. Therefore, more potent antibacterial or antioxidant compounds that are nonpolar and hence insoluble in water may be separated in the extraction process.

Conclusion:

Overall, earthworm extract has strong antibacterial properties as it displayed bactericidal properties on Gram-negative bacteria. Earthworm extract also displayed strong antifungal properties as it reduced the fungal growth by a large percentage. However, earthworm extract

displayed weak antioxidant properties. Since earthworm extract is a weak antioxidant, it is not logical to use it for wound healing purposes as a more common antioxidant(vitamin c) could be used. However, an antiseptic solution could be developed using active compounds of earthworm extracts and applied on chronic wounds. This will mitigate the problem of wound infections.

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