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# Antibacterial Activity of *Lumbricus Rubellus* Earthworm Extract Against *Porphyromonas Gingivalis* as the Bacterial Cause of Periodontitis

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**Abstract**

**AIM:** The purpose of this study was to determine the antibacterial activity of *Lumbricus rubellus* earthworms through inhibitory zone diameter to the growth of the bacterium *Phorphyromonas gingivalis* as the cause of periodontitis.

**METHODS:** This was an experimental study with randomised posttest-only control group design. The study was conducted at the Microbiology Research Center laboratory at the Faculty of Dentistry, Airlangga University, Indonesia. The study was conducted in vitro, the sample size was calculated using the Federer formula as many as four agar plates containing bacteria *Phorphyromonas gingivalis*, with each plate given five different treatments: control (ethanol), *Lumbricus rubellus* earthworm extract (ECT) with concentrations of 50%, 25%, 12.5%, and 6.25% respectively. The data in the form of inhibition zone diameter (measured in millimetres) obtained were tested using One-Way ANOVA.

**RESULTS:** The mean diameter of the inhibitory zone extract of *Lumbricus rubellus* earthworm on the growth of Phorphyromonas gingivalis bacteria in the treatment group had significant differences ($p < 0.05$). The mean inhibition zones between controls and the ECT treatment group (ECT 50%, ECT 25%, ECT 12.5%) were statistically different ($p < 0.05$), in contrast with ECT 6.25% ($p > 0.05$) which did not show significant difference with the control group ($p > 0.05$).

**CONCLUSION:** *Lumbricus rubellus* earthworm extract with a concentration of 50% has the largest diameter of the inhibitory zone on the growth of the *Phorphyromonas gingivalis* bacteria. The 6.25% earthworm extract showed no antibacterial activity against the growth of *Phorphyromonas gingivalis* bacteria.

## Introduction

Periodontal disease is an inflammation that involves the gingiva and alveolar bone. It is one of the most widespread diseases in society, with the most common forms of the disease are gingivitis and periodontitis [1]. Periodontitis is defined as inflammation or infection of the tooth-supporting tissues including gingiva, alveolar bone, periodontal ligament, and cementum. Periodontitis may develop from untreated gingivitis. The disease will spread from the gums towards the bone below the teeth, causing more damage to the periodontal tissue [2]. Microbiological factors are one of the causes of periodontitis. Periodontitis occurs due to unbalanced conditions between host and bacteria, caused by a decrease in host conditions and increased plaque biofilm and bacterial virulence [3]. Specific *Phorphyromonas gingivalis* microorganisms are often detected in patients with periodontitis. These bacteria can be detected in periodontally healthy subjects in the subgingival sulcus region as they can be part of the normal flora of many individuals. These bacteria do not ferment carbohydrates. Their lives depend on amino acid fermentation as energy production. The absolute requirement for the growth of this bacterium

is iron. It is a Gram-negative bacterium in the form of an obligatory anaerobic, non-motile, asaccharolytic stem, which forms pigmented black colonies on a blood agar plate [4].

The degree of periodontal damage depends on the balance between the damaging and protective inflammatory mediators. The primary goal of periodontitis therapy is to maintain the integrity of the teeth by achieving and maintaining healthy periodontium function. It consists of motivation and oral hygiene instructions and mechanical supra and subgingival plaque removal and calculus deposits, plaque correction as the main factor and risk factor (e.g., smokers). Debridement on the root surface of the tooth using scaling and root planning is relatively commonly used in periodontal therapy. Certain patients sometimes do not respond well to conventional mechanical treatment; for various reasons the use of antimicrobials as an adjuvant may be beneficial to patients. Antimicrobials are chemotherapeutic agents that reduce the number of bacteria present in certain surface organisms or by cutting all bacteria. A systematic review by Mathur et al. was conducted to update scientific evidence about antimicrobial properties in addition to subgingival debridement in the treatment of chronic periodontitis [5]. Local application of chlorhexidine and metronidazole locally shows minimal effects; this systematic review shows that scientific evidence supports the use of antimicrobials, especially when carrying out conventional therapy. Antimicrobials are the basis for the treatment of microbial infections, but irrational use is a significant factor in the resistance of microorganisms to antimicrobials [6]. The purpose of irrational antimicrobials will cause unexpected resistance and side effects. *Lumbricus rubellus* earthworm is one of the natural ingredients which has the ability as an antimicrobial.

*Lumbricus rubellus* contains an antimicrobial peptide (AMP) called Lumbricin-1 which functions as a natural defence against pathogenic microbes [7]. Subsequent research was conducted by Rinanda et al., who compared the antimicrobial activity of deep spectrum *Lumbricus rubellus* powder to several microbial resistant drugs such as *Multidrug-Resistant* (MDR) immune to *Pseudomonas aeruginosa*, *Methicillin-Resistant Staphylococcus* aureus (MRSA) and Fluconazole resistant to *Candida albicans*. Statistical analysis showed that *Lumbricus rubellus* powder in the concentration tested had significant antimicrobial activity in a broad spectrum of microbial resistant bacteria [8]. Research on anti-microbial activity was also carried out by Istiqomah *and* her colleagues regarding the inhibition of *Lumbricus rubbellus* worm granule extract against pathogenic bacteria *Escherichia coli, Salmonella Pullorum, Pseudomonas aeruginosa* and *Staphylococcus aureus* in vitro. The study used two types of *Lumbricus rubellus* (ECT) worm extract, dry worm extract (ECT-k) and granular worm extract (ECT-g). The results showed no antibacterial activity from ECT-g to E coli. The diameter of the 24-hour inhibition zone showed that *S aureus* was the most sensitive bacteria to ECT and ECT-k and *S pullorum* most sensitive to ECT-g [9].

Based on the various antimicrobial activities exhibited by studies mentioned above, the purpose of this study was to observe the antibacterial activity of *Lumbricus rubellus* earthworm extract through the inhibitory zone diameter of the bacterium *Phorphyromonas gingivalis* as a cause of periodontitis.

## Material and Methods

This study was an experimental study in vitro with a randomised posttest-only control group design, conducted at the Microbiology Research Center laboratory at the Faculty of Dentistry, Airlangga University, Indonesia. The sample size was calculated by Federer formula as many as four agar plate for *Phorphyromonas gingivalis* bacteria replication. This study was designed using 5 different treatment groups: control (ethanol), extracts of *Lumbricus rubellus* earthworm (ECT) with different concentrations: 50%, 25%, 12.5%, and 6.25% [10] . The data in the form of inhibition zone diameter (millimetres) obtained were tested with One-way ANOVA. The technical procedures were as follow:

1. Earthworm flour production

The Earthworm flour was obtained from *Bali Organic Association (BOA)* worm farming, Denpasar, Bali, Indonesia. Earthworms were separated from the culture media and fasted for 6 hours. The dirt from the digestive worms was removed and washed with water. The worm bodies were soaked with distilled water 6-8 hours, then dried. Soaked worm bodies were being put in the oven with a constant temperature of 40°C for three days, then ground with a blender until it became a flour-like consistency.

2. Earthworm extract production

The earthworm extract was made by the maceration method using 1 kg of earthworm flour dissolved with 3500 ml ethanol. The solution was stirred and soaked for 24 hours, then filtered with *Whatman* paper to separate the filtrate and residue. A total of 1500 ml of filtrate was obtained, then evaporated with a rotary evaporator to obtain 15,570 grams of earthworm extract [11].

3. Culture media for *P. gingivalis* bacteria

The agar media was made using HHI-enriched BHI-A with vitamin K. The components needed to make 100 ml BHI-A were 50 µl hemin solution, 10 µl vitamin K, BHI-A 37 g in 100 ml sterile

aquades and 500 µl yeast extract. The media was divided into four Petri dishes and then be awaited until they became solid. One bacterial use from the ATCC 33277 bacterial stock was inoculated and then incubated at 37°C for 24 hours [12].

4. Preparing the *P gingivalis* bacteria suspension

The bacterial suspension was made by incorporating one use of *P gingivalis* bacteria from BHI-A into a liquid media with total volume of 10 ml containing 0.37 g BHI-B, 1 µl vitamin K, 5 µl hemin and 50 µl yeast extract. The suspension was then incubated for 24 hours, and the concentration was measured to obtain turbidity equivalent to 1.5 x $10^6$ CFU/ml [12].

5. Planting the suspension of *P gingivalis* bacteria.

The *P gingivalis* suspension was swabbed on the surface of the agar media. The even distribution was made possible due to the nutrient contents in the suspension. The paper discs containing different concentrations of ECT, 50%, 25%, 12.5% , and 6.25% respectively were placed on the agar surface, then incubated at 37°C for 24 hours [13].

6. The area without visually apparent bacterial growth (clear zone) around each disc was observed. The diameter of the clear zone was measured using a calliper [13].

## Results

The results of the research on the inhibitory capability of *Lumbricus rubellus* earthworm extract on the growth of *Phorphyromonas gingivalis* bacteria can be seen in Figure 1, below:

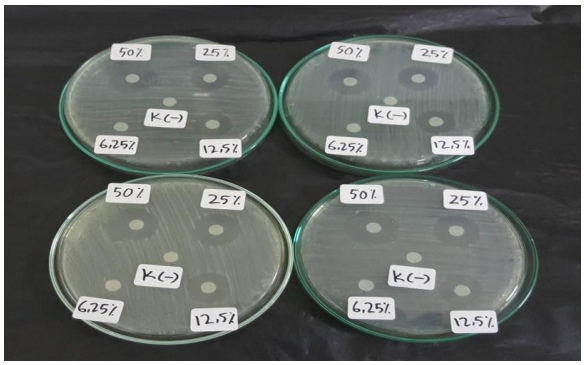


*Figure 1: The inhibitory zone of Lumbricus rubellus earthworm extract (in millimetres)*

The mean ECT inhibition zone diameter of the *Phorphyromonas gingivalis* bacteria in the treatment group was tested using One-Way ANOVA as in Table 1:

**Table 1: The width of *Phorphyromonas gingivalis* Inhibitory Zone in The Treatment Groups**

| Subject Group | n | Mean ± SD | *P** |
|---|---|---|---|
| | | *P. gingivalis* inhibition zone (millimetres) | |
| Control | 4 | 0.00 ± 0.00 | |
| ECT 50% | 4 | 21.88 ± 0.55 | |
| ECT 25% | 4 | 17.23 ± 0.54 | 0.001 |
| ECT 12.5% | 4 | 15.50 ± 0.42 | |
| ECT 6.25% | 4 | 0.00 ± 0.00 | |

Note; Analysis with One-Way ANOVA Test; * significant if $p < 0.05$.

The result from Table 1. shows that the mean diameter of the inhibitory zone of the ECT had significant differences ($p < 0.05$).

**Table 2: The difference of *Phorphyromonas gingivalis* Inhibitory Zone Between the Treatment Groups**

| Group | Mean Diff. | *P** |
|---|---|---|
| Control - ECT 50% | 21.88 | 0.001 |
| Control - ECT 25% | 17.23 | 0.001 |
| Control – ECT 12.5% | 15.50 | 0.001 |
| Control – ECT 6,25% | 00.00 | 1.000 |
| ECT 50% - ECT 25% | 04.65 | 0.001 |
| ECT 50% - ECT 12.5% | 06.38 | 0.001 |
| ECT 50% - ECT 6.25% | 21.88 | 0.001 |
| ECT 25% - ECT 12.5% | 01.73 | 0.001 |
| ECT 25% - ECT 6.25% | 17.23 | 0.001 |
| ECT 12.5% - ECT 6.25% | 15.50 | 0.001 |

Note; Analysis with *Post Hoc Test*; * significant at $p < 0.05$.

Table 2 shows the mean difference of the inhibitory zone between controls and the ECT treatment groups. The mean differences between ECT 50%, ECT 25%, ECT 12.5% groups, and control group were statistically significant ($p < 0.05$), but there was no significant difference between ECT 6.25% and control group ($p > 0.05$).

## Discussion

Antibiotics and antimicrobial compounds inhibit the growth of microorganisms such as bacteria, fungi, and yeast. A zone of inhibition is the clear area surrounding a sample of an antimicrobial agent that has been deposited on an agar-based culture. Microbial colonies will grow on the agar surface. If the antimicrobial agent is useful, it will produce a clear zone that is free of bacterial growth. The length (or diameter) of the zone of inhibition is measured with a ruler or compiler [13]. The results from Table 1 showed that there were differences in the mean diameter of inhibition produced by extracts of the *Lumbricus rubellus* earthworm at different concentrations. The earthworm extract with a concentration of 50% has the highest mean inhibitory zone measuring 21.88 millimetres, compared to the 25% and 12.5% concentration which were 17.23 and 15.50 millimetres respectively. At the concentration of 6.25%, the earthworm extract did not cause inhibition for the growth of *Phorphyromonas gingivalis* bacteria, where the average diameter of the inhibition zone was the same as ethanol control which was 0 millimetres. The width of the inhibitory zone appeared to decrease with fewer concentrations of *Lumbricus rubellus*

earthworm extracts. The difference in the diameter of the inhibitory zone of *Lumbricus rubellus* among the treatment groups as portrayed in Table 2 showed that there was a significant difference between the control and *L. rubellus* earthworm extract at the concentration of 50%, 25% and 12.5% ($p < 0.05$). In the *L. rubellus* earthworm extract group at the concentration of 6.25%, when being compared to the control group, there was no statistically significant difference ($p > 0.05$). In the group of *L. rubellus* earthworm extract concentration of 50% compared to the treatment group at the concentration of 25%, 12.5%, and 6.25% there were significant differences ($p < 0.05$). There were significant differences ($p < 0.05$) between the mean inhibitory zone in the treatment groups of *L. rubellus* earthworm extract of 25% concentration and the 12.5% and 6.25% concentration groups. Similarly, there was a significant difference between treatment groups extract of earthworms at the concentration of 12.5% and 6.25% ($p < 0.05$).

The results of this study prove that earthworm extract can inhibit the growth of the bacteria *Phorphyromonas gingivalis*. Earthworm extract has long been used as a food ingredient and treatment for Ayurveda, Traditional Chinese Medicine, Vietnam, Japan, and Korea [14]. Earthworms have unique properties for treatment including anti-inflammatory, anti-oxidant, anti-tumour and anti-bacterial [15]. Cooper et al., found the role of earthworms on bacterial lysis and their implications on other diseases through lysenin and eiseniapore molecules. Binding of lysenin to sphingomyelin at the cellular membranes serves as a useful tool in investigating the function of sphingomyelin in biological membranes and in explaining the bacterial lysis mechanism of the earthworms. This mentioned lysis pathway might explain the earthworm extract's contribution to the defensive immune system against bacteria. Some studies have shown that earthworms are a source of antibacterial agents that earthworms as land occupants have a robust survival mechanism. The defence of earthworms to protect themselves against attacks by pathogenic organisms has various immune mechanisms by producing granular amoebocytes, chloragocytes and hyaline. Granular amoebocytes and hyaline help in the process of phagocytosis, while chloragocytes produce extracellular products that are cytotoxic and antibacterial [14]. Another study determining the capability of *Lumbricus rubellus* earthworm extract as an antibacterial agent calculated the *minimum inhibitory concentration* (MIC) and *minimal bactericidal concentration* (MBC). It was found that earthworm extract had a strong bactericidal effect against *Shigella flexneri*, and was bacteriostatic against *Streptococcus beta hemolytic* bacteria and *Vibrio cholera* [16].

The *Lumbricus rubellus* earthworm also contains an antimicrobial peptide (AMP) called Lumbricin-1 which functions as a natural defence against pathogenic microbes in the environment [7]. This proline-rich peptide is expressed constitutively by adult worms from *L rubellus* and has shown a broad spectrum of antimicrobial peptides to gram-positive and negative bacteria and fungi [17]. Lumbricin-1 has a hydrophobic surface formed by hydrophobic amino acids. Lipid bilayers on bacterial cell membranes have a hydrophilic and hydrophobic surface. The antimicrobial mechanism could be described as the hydrophobic surface of the peptide interacts with the hydrophilic surface of the cell membrane which results in increased permeability of the cell membrane. Therefore lumbricine-1 can enter the hydrophilic lipid layer.

Furthermore, lubricant-1 enters the hydrophobic layer, where lubricant-1 can adjust its shape to the cell membrane surface so that cell membranes cannot distinguish foreign peptides. The latter process results in intracellular instability and growth inhibition [18]. Research by Rinanda et al., which aimed to prove the antimicrobial activity of *Lumbricus rubellus* had broad-spectrum effect against *Multidrug-Resistant* (MDR) bacteria *Pseudomonas aeruginosa*, *Methicillin-Resistant Staphylococcus aureus* (MRSA) and fluconazole-resistant *Candida albicans*, obtained significant results ($p < 0.05$) [8].

Earthworm extract, the especially coelomic fluid has the potential to fight against several pathogenic and non-pathogenic bacteria such as *Escherichia coli, Streptococcus pyogenes*. In particular, coelomic liquid earthworm *Eisenia Andrei* displays antibacterial properties against *Bacillus megaterium*. The G-90 protein, the anti-carcinogenic molecule mentioned earlier, also has proven antibacterial activity against *Strep. Pyogenes, P aeruginosa*. By using new sources of antimicrobial substances such as the G-90, it seems that high activity of certain earthworm products can be tested to restrain or control microbial threats. These findings prove that effective earthworm extract as an antimicrobial agent against various bacteria only shows one of the many potential benefits of earthworms as therapeutic agents [14].

In conclusion, *Lumbricus rubellus* earthworm extract with a concentration of 50% has the largest diameter of the inhibitory zone against the growth of *Phorphyromonas gingivalis* bacteria. The 6.25% earthworm extract showed no antibacterial activity against the growth of *Phorphyromonas gingivalis* bacteria. These findings can be used as a basis for periodontitis therapy by using *Lumbricus rubellus* earthworm extract.

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## Author Contributions

All authors had contributed in providing suggestions and thoughts in this research by their respective disciplines, thereby doing this research ran with no significant hindrance and was subsequently achieved in obtaining results following the research objectives.

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