# BROAD SPECTRUM ANTIMICROBIAL ACTIVITY OF *LUMBRICUS RUBELLUS* POWDER AGAINST DRUG RESISTANT MICROBES

[1]TRISTIA RINANDA, [2]SAKDIAH, [3]RISNA OKTAVIA, [4]MEYCHA DA FHONSA AND [5]MUFQI FITRA

[1]Department of Microbiology, Faculty of Medicine, Syiah Kuala University, Banda Aceh 23111, Indonesia; [2]Department of Biochemistry, Faculty of Medicine, Syiah Kuala University, Banda Aceh 23111, Indonesia; [3]Clerkship student, Faculty of Medicine, Syiah Kuala University-Dr.Zainoel Abidin Hospital, Banda Aceh
Corresponding Author: tristia_rinanda@yahoo.com

## ABSTRACT

Inappropriate uses of antimicrobial drugs have contributed to the development of resistant microbial pathogens. This phenomenon requires discovery and development of potential antimicrobial compounds from organic materials. *Lumbricus rubellus* is the ubiquitous earthworm containing antimicrobial peptide named Lumbricin-1. The aim of this study was to determine the broad spectrum antimicrobial activity of *Lumbricus rubellus* powder against several drug resistant microbes such as Multidrug Resistant (MDR) *Pseudomonas aeruginosa*, Methicillin Resistant *Staphylococcus aureus* (MRSA) and Fluconazole resistant *Candida albicans.* This experimental study was conducted using Completely Randomized Design (CRD) with 6 treatment groups consisting of 100 mg, 200 mg, 300 mg, 400 mg and 500 mg *L.rubellus* powder in 5 ml solvent (acetic acid and acetonitrile) and negative control groups. One percent of each concentration then was used in the antimicrobial activity testing using Kirby Bauer disc diffusion method. Data of this research were analyzed by ANOVA. The results showed that *L. rubellus* powder in each of the concentrations of 100 mg, 200 mg, 300 mg, 400 mg and 500 mg obtained an average inhibition zone of 14.33 mm, 14.17 mm, 14.00 mm, 15.00 mm and 13.00 mm respectively against MDR *P. aeruginosa*; 16.75 mm, 18.50 mm, 18.75 mm, 16.75 mm and 17.25 mm against MRSA; 16.50 mm, 18.00 mm, 17.50 mm, 17.75 mm and 16 mm against Fluconazole resistant *C. albicans.* Statistical analysis revealed that *Lumbricus rubellus* powder in the tested concentrations showed significant broad spectrum antimicrobial activity against drug resistant microbes ($p<0.05$).

Keywords:Antimicrobial activity, *Lumbricus rubellus*, Lumbricin-1

## Introduction

Antibiotic resistance against microorganisms is still a major problem in the health sector. Resistance to antibiotics is a change in the ability of microorganisms to become resistant to antibiotics. This happens due to the changing nature of the microorganisms that can no longer be inhibited or killed. The drug will lost its efficacy and its potency will decline and even disappear. Microorganisms that are resistant to antibiotics would not be killed by antibiotics, then multiply and spread so that it becomes more dangerous (WHO, 2011). Antimicrobial therapy is the basis for microbial infection (bacteria and fungi), but the irrational use becomes the major factor in the resistance of microorganisms to antimicrobials (Harbottle et al., 2006)

One of the alternatives to encounter the antimicrobial resistance is to use natural materials. The use of these materials, ranging from plants to animals, is increasingly being developed by scientists. One of them is earthworm (*Lumbricusrubellus*) powder. Earthworms (*L.rubellus*) plays an important role in human life because of its high nutrient content, especially protein (64-76%) (Palungkun, 1999).

Earthworms (*L.rubellus*) also contain antimicrobial peptide (AMP) named Lumbricin-1 which serves as a natural defense against microbial pathogens in the environment (Tasiemski, 2008). This proline-rich peptide is expressed constitutively by adult worm of *L. rubellus* and had shown its broad spectrum antimicrobial peptide against Gram positive and negative bacteria and fungi (Cho, et al. 1998). This study aims to determine the broad-spectrum antimicrobial activity of *L. rubellus* earthworm powder against a number of pathogenic microbes isolated from clinical samples such as Multidrug Resistant (MDR) *Pseudomonas aeruginosa*, Methicillin Resistant *Staphylococcus aureus* (MRSA) and fluconazole-resistant *Candida albicans*.

## Materials and Methods

This experimental study was conducted using Completely Randomized Design (CRD) with 6 treatment groups consisting of 100 mg, 200 mg, 300 mg, 400 mg and 500 mg *L. rubellus* powder in 5 ml solvent (acetic acid and acetonitrile) and negative control groups, in normalized concentration (1%).

### *Identification of Tested Microbes*

The tested microbes were isolated from various clinical specimens. The MDR *P. aeruginosa* was isolated from the ear swab of middle ear infection patients. The MRSA isolate was obtained from the *stock culture* isolated from the pus of diabetic ulcer patients and Fluconazole resistant *C. albicans* was acquired from the vaginal swab of vaginal candidiasis patients at the Zainoel Abidin Hospital.

*Macroscopic, Microscopic and Biochemistry test of Identification.* Re-identification of the MDR *P. aeruginosa* was performed using macroscopic (the examination comprised shape, surface, border and color of the colony growing in the Nutrient Agarand Soboroud Dextrose agar medium), and microscopic tests (Gram Staining). The examination was continued by using biochemical identification test using RapID NF Plus (Remel). MethicillinResistant *Staphylococcus aureus* had been identified by using microscopic examination (Gram staining), catalase test and coagulation test. Identification of *C. albicans* was performed by microscopic evaluation (Gram staining, 10 % Potassium Hydroxide examination and germ tube test).

*Test of Resistance.* Inocula was gradually suspended into sterile NaCl 0.9% on the wall of the test tube to produce a fine bacterial suspension. The tested microbes were inoculated to an MHA medium by dipping a sterilized cotton bud into the inocula. The cotton bud was applied onto the surrounding of the agar surface. The inocula was left to dry for a few minutes at room temperature.Resistance test was performed by using oxacillin 5 µg antibiotic disc (for MRSA), Ceftazidime 30 µg, Ceftriaxone 30 µg, Ciprofloxacin 5 µg, Gentamicin 10 µg, Meropenem 10 µg, Tobramicin 10 µg (for MDR *P. aeruginosa*) and Nystatin 100 U and Fluconazole 25 µg (for *C. albicans*) which was incubated at $35^0$C. After that, the diameter of the formed inhibition zone was measured using a vernier caliper and the result was stated in millimeter (mm).

### *The Formulation of Lumbricus rubellus Powder*

Ten kilograms of *L. rubellus* were separated from the enlargement media, then washed and rinsed thoroughly. Next, *L.rubellus* was stored in the refrigerator for 24 hours to detach all dirt. It was thenwashed again and rinsed clean then crushed to form a paste. The paste was heated in the oven at a temperature of 40° C for 48 hours. Dried paste was then pulverized in a blender and filtered. The powder was stored in a sealed container.

***Antibacterial Activity Test of Lumbricus rubellus powder against Drug Resistant Microbes***

The preparation of working solution was based on the peptide dissolution protocol (Thinkpeptide, 2012) with modification. 100mg, 200mg, 300mg, 400mgand500mgearthworm powder were placed in steriletubesthen added 2 mL of 50% acetic acid. The solution then was homogenized by using vortex for 5 minutes. This treatment was repeated twice. Next, 1 mL of Acetonitrilewas added to thesolutiontoachieve atotalvolumeof 5mL and again homogenized. The earthwormpowdersolution used for antimicrobial activity was that with the concentration of 1%.

The antimicrobial activity test applied the Kirby-Bauer disc diffusion method using Mueller Hinton Agar (MHA) medium, in accordance with the procedures of *European Committee on Antimicrobial Susceptibility Testing*/EUCAST (2009). Inocula were suspended gradually into the sterile NaCl 0.9% on the wall of the test tube to produce a fine bacterial and fungal suspension. The turbidity was measured using spectrophotometer at the wavelength of 625 nm and absorbency of 0.08-0.1 for *P. aeruginosa* and *S. aureus*, and 530 nm and absorbency of 0,5-0,6 for *C. albicans*. The tested microbes were inoculated to a MHA medium by dipping a sterilized cotton bud into the inocula. The empty disc were soaked in the 1% powder solution of different concentrations for 30 minutes. It was then set on an inoculated petri dish using sterilized tweezers. The negative control used an empty disc soaked in sterile water. Each treatment was repeated 3 times. The next step was incubation for 24 hours at $37^0$C for bacteria and 48 hours for fungi.

## Results and Discussion

### Identification result of tested microbes

Macroscopic examination showed the growth of oval-shaped colonies of 3 mm in size, with flat edge and smooth surface. The nutrient agar medium turned into green color. The result of Gram staining indicated Gram negative bacilli. The RapID NF-Plus test showed biochemistry characterization of *P. aeruginosa*. The resistance test indicated the bacterial resistance to more than 2 groups of selected antibiotics which refered to MDR (CLSI, 2012).

The result of macroscopic observation on the *Nutrient Agar* (NA) medium showed that *S. aureus* formed a colony of 0.5 mm in size, of white color, round shape with fine border and even surface. Gram staining result showed a bacterium in coccus shape, purple color, and forming a small group resembling grapes which indicated Gram positive bacterium. The coagulation test using plasma in tubes produced a positive result namely the forming of plasma coagulation. From the catalase test, a positive result was obtained which was indicated by the forming of gas bubbles. The result of resistance test of *S. aureus* to *methicillin* showed that *S. aureus* isolates are resistant to antibiotic *methicillin* (Brown, et al, 2005; CLSI, 2012*)*.

Macroscopic examination in Soboroud dextrose agar medium showed the growth of *C. albicans* as smoot or soft colonies of cream coloured, round shape and produced yeast-smell like. The result of Gram staining showed Gram positive coloured of micelium and yeast cells. The true hyphae was showed in germ tube test. All the above mentioned results show that the tested fungi was *C. albicans*. The resistance test indicated the resistance to antifungal Nystatin and Fluconazole (CLSI, 2012).

### Result of Antimicrobial Activity Test of *Lumbricus rubellus* Powder against Drug Resistant Microbes

The antimicrobial test of *L. rubellus* powder against the growth of drug resistant microbes at the 100 mg/5 ml, 200 g/ml, 300 mg/5ml, 400 mg/ml and 500 mg/ml concentrations resulted in inhibition zones of average diameter of 14.33 mm, 14.17 mm, 14.00 mm, 15.00 mm and 13.00 mm respectively against MDR *P. aeruginosa*; 16.75 mm, 18.50 mm, 18.75 mm, 16.75 mm and 17.25 mm against MRSA; 16.50 mm, 18.00 mm, 17.50 mm, 17.75 mm and 16

mm against Fluconazole resistant *C. albicans.* The data obtained was then analyzed using *Analysis of Variance* (ANOVA) to examine the impact of each treatment. The result of ANOVA revealed that there was a significant difference in the antibacterial activity indicated by each concentration of *L. rubellus* powder. Statistical test was then continued with Duncan Test at p<0,05.

Table 1. Average diameter of inhibition zone ± deviation standard of *L. rubellus* powder against the growth of MDR *P. aeruginosa.* The average value of inhibition zone was followed by different superscripts showing significant differences (P<0,05)

| **Treatment** | **Diameter of inhibition zone in each repetition (mm)** | | | | **Average diameter of inhibition zone (mm) ± SD** |
|---|---|---|---|---|---|
| | **I** | **II** | **III** | **IV** | |
| Negative control | 0 | 0 | 0 | 0 | 0± 0,00[a] |
| 100 mg/5 ml | 13 | 15 | 15 | 14,3 | 14,33b ± 1,15 |
| 200 mg/5 ml | 14,5 | 14 | 14 | 14,18 | 14,16[b] ± 0,27 |
| 300 mg/5 ml | 14 | 13 | 15 | 14 | 14,00[b] ± 1,00 |
| 400 mg/5 ml | 15 | 16 | 14 | 15 | 15,00[b] ± 1,00 |
| 500 mg/5 ml | 13 | 13 | 14,5 | 13,5 | 13,5b ± 0,87 |

Table 2. Average diameter of inhibition zone ± deviation standard of *L. rubellus* powder against the growth of MRSA.The average value of inhibition zone was followed by different superscripts showing significant differences (P<0.05)

| **Treatment** | **Diameter of inhibition zone in each repetition (mm)** | | | | **Average diameter of inhibition zone (mm) ± SD** |
|---|---|---|---|---|---|
| | **I** | **II** | **III** | **IV** | |
| Negative control | 0 | 0 | 0 | 0 | 0± 0,00[a] |
| 100 mg/5 ml | 14 | 17 | 18 | 18 | 16,75b ± 1,8 |
| 200 mg/5 ml | 15 | 20 | 19 | 20 | 18,50e ± 2,3 |
| 300 mg/5 ml | 16 | 21 | 18 | 20 | 18,75e ± 2,2 |
| 400 mg/5 ml | 12 | 19 | 17 | 19 | 16,75c ± 3,3 |
| 500 mg/5 ml | 14 | 19 | 18 | 18 | 17,25d ± 2,2 |

Table 3. Average diameter of inhibition zone ± deviation standard of *L. rubellus* powder against the growth of Fluconazole-resistant *C. albicans*.The average value of inhibition zone was followed by different superscripts showing significant differences (P<0.05)

| **Treatment** | **Diameter of inhibition zone in each repetition (mm)** | | | | **Average diameter of inhibition zone (mm) ± SD** |
|---|---|---|---|---|---|
| | **I** | **II** | **III** | **IV** | |
| Negative control | 0 | 0 | 0 | 0 | 0± 0,00[a] |
| 100 mg/5 ml | 17 | 19 | 15 | 15 | 16,5b ± 1,91 |
| 200 mg/5 ml | 17 | 20 | 17 | 18 | 18,0b ± 1,41 |
| 300 mg/5 ml | 16 | 20 | 16 | 18 | 17,5b ± 1,91 |
| 400 mg/5 ml | 19 | 17 | 18 | 18 | 17,7b ± 0,95 |
| 500 mg/5 ml | 16 | 16 | 15 | 17 | 16,0b ± 0,81 |

The presence of inhibition zones showed that earthworm powder *L.rubellus* at any tested concentration obtained antimicrobial activity against MDR *P. aeruginosa*, MRSA and Fluconazole resistant-*C. albicans*. Study performed by Cho, et al, showed that *L. rubellus*

contain antimicrobial peptide named Lumbricin-1, which was able to inhibit the growth of Gram positive and Gram negative bacteria as well as fungi.

Living organisms use antimicrobial peptides as one of the major defense against microbial infection in their environment. Lumbricin-1 isolated from *L. rubellus* (sized 7231 Da, formed by 62 amino acids) is known as proline rich-antimicrobial peptides with broad spectrum antimicrobial activity (Cho, et al 1998). Proline form an unique conformational structure on the peptide chain and affect the secondary structure then define the mechanism of action. In addition to Lumbricin-1, there are other several antimicrobial peptides which are also proline rich such as apidaecins, drosocin, metchikowin, bactenecins and PR-39.

Each antimicrobial perform varied mechanism of action in inhibiting pathogenic microorganisms. Gram negative bacteria are able to be inhibited by apidaecins, bactenecins and PR-39, while drosocin is able to inhibit both Gram positive and Gram negative, but it cannot inhibit the growth of fungi. Gram positive bacteria and fungi can be inhibited by metchikowin, but this microbial peptide has no effect on Gram-negative bacteria. The mechanism of action of Lumbricin-1 is still unclear but one hypothesis states that it demonstrates a different mechanism from other proline-rich antimicrobial peptides. (Cho, et al 1998).

Mechanism of action of antimicrobial peptide begins with the initial interaction between the peptide and the target cell (bacteria or fungi) due to the influence of electrostatic power (Yeaman and Yount, 2003). The level of positive charge (cationicity) plays an important factor for initial electrostatic interaction between antimicrobial peptide and the negatively charged phospholipid membrane of bacteria. The interaction of cations (antimicrobial peptide) and anions (bacteria and fungi cell surface) is alleged to cause changes in the permeability of the cell membrane so that lumbricin-1 can penetrate into the cytoplasmic membrane and cause cell damage (Epand and Vogel, 1999; Jenssen, Pamela and Robert, 2006). There is a strong relationship between the levels of antimicrobial peptide cationicity with antimicrobial activity. The higher cationicity the stronger antimicrobial activity (Yeaman and Yount, 2003).

Lumbricin-1 also has a unique hydrophobic surface feature formed by hydrophobic amino acids. Lipid bilayer on bacterial cell membrane has both hydrophilic and hydrophobic surfaces. In this antimicrobial mechanism, the hydrophobic surface of the peptide interacts with the hydrophilic surface of the cell membrane which consequently increases cell membrane permeability and lumbricin-1 can enter the hydrophilic lipid layer. Furthermore, when entering the hydrophobic layer, allegedly lumbricin-1 has the ability to adjust its shape with the surface of the cell membrane. Therefore, the membrane is unable to distinguish foreign peptides which causes intracellular instability and growth inhibition (Pasupuleti, 2009).

*Candida albicans* has hydrophobic cell wall. It expresses the cell surface hydrophobicity (CSH) which is more virulent compared to cell surface hydrophilicity. Hydrophobic surface plays an important role in attachment to the host's cell, defense against phagocytosis and protection on germination period (Hazen et al, 2001). Hydrophobicity surface is one of the virulent factors in *C. albicans* and it becomes the main target of Lumbricin-1. Lumbricin-1 as antimicrobial peptides has the basic ability to adapt to the structure of the hydrophobic amino acids (Zasloff, 2002). Lumbricin-1 can form a specific secondary structure and hydrophobic surface molecules (Cho et al, 1998). The presence of negative charge on the cell wall of *C. albicans* in addition to hydrophobicity allowed greater interaction with antimicrobial peptides (Zasloff, 2002). The broad spectrum antimicrobial activity demonstrated by *L. rubellus* powder indicates that antimicrobial peptide Lumbricin-1 isolated from this organism can be potentially developed as a potent antimicrobial agent.

The presence of Lumbricin-1 in *L. rubellus* powder should be confirmed by using Tricine SDS-PAGE. This important step could not be performed due to unavailability of Tricine.

## Conclusions

The *L. rubellus* powder at any tested concentration showed broad spectrum antimicrobial activity against MDR P. aeruginosa, MRSA and Fluconazole-resistant *C. albicans*. The antimicrobial activity muncul diduga akibat adanya antimicrobial peptide Lumbricin-1. Furthermore, based on the results of this study, earthworm antimicrobial peptide Lumbricin-1 can be developed as a promising antimicrobial agent.

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