Identification and antimicrobial activity test of endophytic fungi from water hyacinth petiole (*Eichhornia crassipes*) against *Escherichia coli* and *Staphylococcus aureus*

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Abstract. Endophytic fungi have bioactive compounds in the form of secondary metabolites that come from reciprocal relationships with their host plants. Microorganisms such as endophytic fungi have important role in the production of bioactive compounds in the field of biotechnology. The main goal of this study was to find out the antimicrobial activity of endophytic fungi from water hyacinth petiole against *E. coli* and *S. aureus*. The endophytic fungi were cultured from petiole of water hyacinth on PDA for 4-5 days at 30ºC. The fungi isolates that have grown were purified and used to test of antimicrobial activity. The method used for antimicrobial test is overlay and disc diffusion agar test. There were 8 endophytic fungi isolated, a total of 4 endophytic fungi identified. Overlay test of the results showed only three isolates had antimicrobial activity i.e. *Aspergillus* sp., *Trichophyton* sp., and *Penicillium* sp. Disk diffusion test of the results showed that *Penicillium* sp. had strong antimicrobial activity with inhibition zones 13.6 ± 1.4 mm (*E. coli*) and 7.5 ± 1.1 mm (*S. aureus*). Meanwhile, *Aspergillus* sp. produced antimicrobial activity with inhibition zones 2.5 ± 0.2 mm and *Trichophyton* sp. of 2.8 ± 0.7 mm against *E. coli* bacteria.

1. Introduction

Infectious disease is a disease that most affects people in developing countries, belonging Indonesia. The disease is caused by microbial contamination in human life [1]. *Escherichia coli* and *Staphylococcus aureus* are pathogenic bacteria that caused infections on humans. *E. coli* is a bacterium that causes digestive tract diseases (*cholera*) and dysentery against children and adults [2]. Meanwhile, *S. aureus* could cause tissue damage due to abscesses like carbuncle, pimple, impetigo, and skin wound infections [3].

Antibiotics were used to cure diseases caused by bacterial infections. However, extreme usage of antibiotics and long periods of time can cause resistance to bacteria and have side effects in their use [4]. Water hyacinth is a plant can be used to source of new antimicrobials. The influence of polluted aquatic environments causes microorganisms that live in water hyacinth hold specific secondary metabolites [5].

Endophytic fungi are microorganisms that live in all water hyacinth tissues, appertain leave, petiole, root, and flower. All of these tissues have different metabolite compound. Petiole tissue has metabolite compound will be used by endophytic fungi in breaking down contaminants that have complex molecular chains into simple molecules [6]. The mutualism symbiosis of endophytic fungal
by received protection and nutrition from the host, while the host plant gets the benefit of protection against natural enemies such as pathogenic microorganisms and herbivorous animals [7]. The population of endophytic fungi in water hyacinth is strongly influenced by climatic conditions and location of the growth of host plants so that endophytic fungi can produced a new antimicrobial compound [8].

2. Materials and methods

2.1. Place and time
This research was conducted from December 2019 until March 2020 in the Microbiology Laboratory of the Faculty of Fisheries and Marine, Airlangga University in Surabaya, Indonesia.

2.2. Tools and materials research
The tools used in this study were petri dishes, test tubes, erlenmeyers, glass beakers, measuring cups, loop ose, knives, objects and glass cover, masking tape, microtube, vortex, micropipette, hotplate, microscope, refrigerator, analytical balance, laminary air flow, autoclave and incubator.

The materials used were a physiological NaCl, BaCl₂ solution 1%, H₂SO₄ 1%, alcohol 70%, agarose 1.5%, aquades, Potato Dextrose Agar (PDA) and Broth (PDB), Tripticase Soya Agar (TSA), lactophenol blue, and Mueller-Hinton Agar (MHA).

2.3. Work procedures

2.3.1. Tool and materials preparation
Tools were prepared namely petri dish, test tubes, Erlenmeyers, beaker glass, and microtubes wrapped in heat-resistant plastic. Media for fungi growth was made by mixing media powder with distilled water. After that, it was sterilized using an autoclave at 121°C for 15 minutes.

2.3.2. Isolation and purification
The plant of E. crassipes were collected from river in Wonoayu, Sidoarjo, East Java. The petioles are washed with tap water for 3 minutes to remove the sludge and debris. Surface sterilization was done by sequentially dipping the petiole in 70% alcohol for 5 s followed by rinsing them in sterile distilled water and further dried with tissue. Then, the petiole was dried up in laminary air flow for 5 hours [9].

Sterile petioles were cutted use a sterile knife with a size of 1 cm x 1 cm horizontally and then placed on Potato Dextrose Agar (PDA) which has been added with 100 µg / mL chloramphenicol. The plates were incubated at 30°C for 3-4 days. The endophytic fungi obtained were inoculated to the new PDA media by pricking and incubated at 30°C for 3-4 days more to get produced pure (single) isolates [25].

2.3.3. Identification of endophytic fungi
Endophytic fungi that have been obtained are then macroscopically and microscopically identified. Macroscopic identification was done through observing the morphology and color of the colony that grows on agar media. Meanwhile, Microscopic identification was use a binocular microscope to observing at spores by lactophenol cotton blue staining [10].

2.3.4. Fermentation and extraction
Fermentation of endophytic fungi was done by liquid fermentation on Potato Dextrose Broth (PDB) media. The pure endophytic fungi colonies that have grown on PDA petri dish was taken 1 section of fungal culture with the dimensions of ± 1 x 1 cm to the 20 ml PDB media. After that, incubation was done using a 100 rpm of incubator shaker at 30°C for 7 days. The fermented of endophytic fungi was put into a 15 ml sterile centrifuge tube, then centrifuged at 3000 rpm for 20 minutes. Supernatant was taken and used to antibacterial activity test [11].
2.3.5. **Antimicrobial activity**

Antimicrobial activity test using the overlay method and disk diffusion agar. Overlay method is a qualitative bacterial testing method to observe the antibacterial activity. The test bacterial isolates with standard Mc Farland 1 turbidity were taken 200 µl and put into an Erlenmeyer containing 20 ml of MHA soft agar. After that, the mixture was poured into fungi isolates that had grown on PDA media, and incubated at 30ºC for 24 hours. Positive results was marked by the formation of clear (inhibitory) zones around the fungal colonies after incubating [12].

Disk diffusion agar method is a quantitative method as an antimicrobial further test. Each paper disc was filled with 30 µl of the endophytic fungal supernatant strain, allowed to stand for 15 minutes so that the paper disk absorbed the supernatant. Then, placed on the surface of the MHA media cup which already contains bacterial isolates, the test was maked in two replications [11]. The positive control was used a 30 µg chloramphenicol disc and for the negative control was used sterile blank disc. After disk placement, it was incubated at 37ºC for 18-24 hours [13]. The measurement of inhibition zone diameter is done by reducing the diameter of the obstacle area to the diameter of the paper disc using a crossbar.

2.3.6. **Data analysis**

Data from the results of this study were analyzed descriptively and presented in tables and figures. Macroscopic and microscopic observational data will be used for identification based on the Identification of Pathogenic Fungi handbook [14] and the Laboratory Identification of Pathogenic Fungi Simplified [15].

3. **Results and discussion**

3.1. **Result**

The results of identification of endophytic fungi from water hyacinth petiole managed to get 8 isolates were purified. Details of the identification results by endophytic fungi obtained are endophytic fungi species *Aspergillus* sp., *Curvularia* sp., *Penicillium* sp., and *Trychophyton* sp. The results of identification of endophytic fungi can be seen in Table 1.

| No. | Isolate code | Endophytic fungi species     |
|-----|--------------|------------------------------|
| 1.  | JE.1         | *Penicillium* sp.            |
| 2.  | JE.2         | *Aspergillus* sp.            |
| 3.  | JE.3         | *Trychophyton* sp.          |
| 4.  | JE.4         | *Aspergillus* sp.            |
| 5.  | JE.5         | *Penicillium* sp.           |
| 6.  | JE.6         | *Aspergillus* sp.            |
| 7.  | JE.7         | *Curvularia* sp.             |
| 8.  | JE.8         | *Aspergillus* sp.            |

JE : endophytic fungal isolate code

The results of identification by macroscopically of *Aspergillus* sp. visible blackish brown colony, yellow, and brownish yellow. The colony grows broadly, had a granular, flat, and piled surface. *Aspergillus* sp. colony can be seen Figure 1. Meanwhile, microscopic identification showed conidia radial heads, fialids grown in all segments of vesicles and conidia piled up. The spore formed can be seen Figure 2.
The results of identification by macroscopically of *Curvularia* sp. was colony rounded, color black of colony with dark green edges, and flat topography. *Curvularia* sp. colony can be seen Figure 3. Meanwhile, microscopic identification showed conidia form curved (rounded elongated), hyphae with septa and branched, conidia composed of several septa. The spore formed can be seen Figure 4.

The results of identification by macroscopically of *Penicillium* sp. were colony gray, black green with white edges, rough colony texture. *Penicillium* sp. colony can be seen Figure 5. Meanwhile, microscopic identification showed conidia is oval in shape with both asymmetrical ends, small, smooth, and branched conidiophores. The spore formed can be seen Figure 6.
The results of identification by macroscopically of *Trychophyton* sp. was colony grows large and wide rapidly, color of the front colony was grayish white, reverse colony was creamy, flat topography. *Trychophyton* sp. colony can be seen Figure 7. Meanwhile, microscopic identification showed conidiophore were smooth, septated, and branched. small conidia (micro), and branched hyphae. The spore formed can be seen Figure 8.

The results of the overlay method antimicrobial activity test succeeded in getting three positive endophytic fungi isolates that were able to inhibit the bacteria growth of *E. coli* (ATCC 8739) and *S. aureus* (ATCC 6538). Positive results are marked with a clear zone (clear) that is around the endophytic fungi isolate colonies that grow. Overlay method results can be seen in Table 2. There were 2 endophytic fungi isolates which are able to inhibit the growth of one *E. coli* pathogenic bacterium namely endophytic fungi isolates JE.3 and JE.4. Endophytic fungal isolates from JE.5 can inhibit the growth of two bacteria *E. coli* and *S. aureus*. Whereas the other endophytic fungi isolates are negative because they have no clear zone. The results of the overlay test can be seen in Figure 9 and Figure 10.
Table 2. Antimicrobial activity test results with overlay method

| No. | Isolate | Antimicrobial activity |  |  |
|-----|---------|------------------------|---|---|
|     |         |                        | **E. coli** | **S. aureus** |
|     |         |                        | ATCC 8739 | ATCC 6538 |
| 1.  | JE.1    | -                      | -           | -           |
| 2.  | JE.2    | -                      | -           | -           |
| 3.  | JE.3    | +                      | -           | -           |
| 4.  | JE.4    | +                      | -           | -           |
| 5.  | JE.5    | +                      | +           | +           |
| 6.  | JE.6    | -                      | -           | -           |
| 7.  | JE.7    | -                      | -           | -           |
| 8.  | JE.8    | -                      | -           | -           |

JE : endophytic fungal isolate code
(+): Isolate endophytic produced clear zone,
(-): Isolate endophytic not produced clear zone

Three endophytic fungi isolates i.e Aspergillus sp. Penicillium sp. and Trychophyton sp. were have a clear zone (inhibitory) in the overlay test, carried out further tests was disk diffusion agar method. Disk diffusion test results should be shown in Table 3. Antimicrobial activity in the largest disk diffusion test was shown by JE.5 isolates against E. coli bacteria by 13.6 ± 1.4 mm and S. aureus by 7.5 ± 1.1 mm. While the antimicrobial activity of the other two isolates against E. coli is JE.3 of 2.8 ± 0.7 mm and JE.4 of 2.5 ± 0.2 mm. Chloramphenicol 30 µg as a positive control has a inhibition zone of 30 ± 0.5 in E. coli bacteria and 27 ± 0.5.
Table 3. Antimicrobial activity test results with disc diffusion agar method

| Isolate | Endophytic fungi inhibition zone (mm) |
|---------|--------------------------------------|
|         |  **E. coli** ATCC 8739  | **S. aureus** ATCC 6538  |
| JE 3    | $2.8 \pm 0.7$                | $0 \pm 0$                |
| JE 4    | $2.5 \pm 0.2$                | $0 \pm 0$                |
| JE 5    | $13.6 \pm 1.4$               | $7.5 \pm 1.1$            |
| Control (+) | $30 \pm 0.5$            | $27 \pm 0.5$            |
| Control (-) | $0 \pm 0$               | $0 \pm 0$               |

JE : endophytic fungal isolate code
($\bar{x} \pm SD$) : mean of inhibition zone with standard deviation

3.2. Discussion

The results of identification of endophytic fungi from water hyacinth petiole managed to get species *Aspergillus* sp., *Curvularia* sp., *Penicillium* sp., and *Trychophyton* sp. *Aspergillus* sp. macroscopically it has the characteristics of a fast grower colony, the colors of the colonies are white, yellow, brownish yellow, green, and blackish brown. Microscopically, the ends of the conidiophores form vesicles that contain a metula structure, covered with fialids which produce conidia [15]. *Curvularia* sp. has a dark brown or black gray colony, the back of the colony is black, flat topography [14]. Microscopically, conidia were usually composed of 4 septa, asymmetrical cells form from both ends of the cell (*penultimate cell*) and elongated [15]. *Penicillium* sp. macroscopically has the characteristics of a colony that is fast grower, flat, turquoise with a rough surface with a white points. Microscopically, structure consisted of chains or spores which were clamped from pumpkin-shaped sterigmata (phialids) with the points of the metulae, short branched or unbranched. Conidia were small, smooth and ellipsoidal in shape that extends like chains [15]. *Trichophyton* sp. has a white colony, grayish white, cream, cream rear colony, brownish cream, and pink, granular texture, and flat topography.
Microscopically, *Trichophyton* sp. has a spiral and smooth hyphae form, consisted of microconidia and macroconidia, has hyphal branched, microconidia grown at the tip or the end segment of the hypha branch [14].

The results showed that of endophytic fungal species *Aspergillus* sp. and *Penicillium* sp. more found. Endophytic fungi species *Aspergillus*, *Phomopsis*, *Wardomyces*, and *Penicillium* were generally found in plants that live in tropical and subtropical regions [16]. Endophytic fungi isolated from water hyacinth petiole can inhibit the growth of pathogenic bacteria by forming clear zones on agar media. Endophytic fungi were can formed clear zones caused by antimicrobial compounds produced. Antimicrobial compounds of endophytic fungi derived from secondary metabolites produced through biosynthesis that are different from their host [17]. Endophytic fungi were capable of producing secondary metabolites to protect host plants from attack by harmful organisms. Secondary metabolites produced by endophytic fungi include terpenoids, phenols, xanthones, steroids, isocumarin, cytokalasin, tetralon, benzopyran, and enniatin [18].

*Trichophyton* sp. was isolated from the soil at a disposal site, produces the enzyme keratinase. Keratinase has the ability to remodel the structure of cell wall chemical tissue, the breaking of hydrogen bonds and disulfide bonds that produce proteins, lipids, and amino acids [19]. *Aspergillus* sp. has alkaloïd compounds that can inhibit bacterial growth. Alkaloids work as antibacterial by damaging the components of peptidoglycan in bacterial cells [20]. *Penicillium* sp. can produce bioactive compounds in the form of penicillin in killing or stopping bacterial growth. The mechanism of action of penicillin by preventing peptidoglycan crosslinking in the final stages of cell wall synthesis is by inhibiting penicillin binding protein [21].

Supernatant endophytic fungi isolates were less effective when compared to the chloramphenicol used. However, the used of chloramphenicol antibiotics in the long term and excessive cause resistance to bacteria [22]. The use of chloramphenicol as a positive control because it was an antibacterial that’s bacteriostatic (inhibits bacterial growth) on a extensive spectrum, both Gram-negative and Gram-positive bacteria [23].

The antimicrobial activity of endophytic fungi has differences in fighting pathogenic bacteria. Antimicrobial activity was influenced by hyphal growth, nutrient content in the growth site, and secondary metabolite compounds produced by endophytic fungi specific [5]. This difference was also influenced by the structure of Gram-positive and Gram-negative bacterial cell walls. Gram-negative bacteria have a thick layer of peptidoglycan with a low lipid content, while Gram-negative bacteria have a layer consisting of lipoproteins, lipopolysaccharides, and peptidoglycan. Peptidoglycan Gram-negative bacteria are thin and contain lots of lipids. These differences cause the performance of antimicrobial compounds to experience differences in binding and penetrating into the bacterial cell wall [24].

### 4. Conclusion

Based on research on the identification and testing of endophytic fungal antimicrobial activity of water hyacinth petiole against *E. coli* and *S. aureus* bacteria, it can be concluded that there are three endophytic fungal isolates that have antimicrobial activity, namely *Aspergillus* sp. and *Trichophyton* sp. which is able to inhibit the bacteria *E. coli*, and *Penicillium* sp. which is able to inhibit the growth of two bacteria namely *E. coli* and *S. aureus*. Strong antimicrobial activity was found in endophytic fungi of *Penicillium* sp.

### 5. References

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