The potential of isolates endophytic fungi from the leaves of henna plant (Lawsonia inermis) toward the growth of Candida albicans and Staphylococcus aureus

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Abstract. This study was aimed to investigating the influence of isolates endophytic fungi from the leaves of the henna plant (Lawsonia inermis) toward the growth of Candida albicans and Staphylococcus aureus and finding out the significant differences of each isolate endophytic fungi treatment toward the growth of C. albicans and S. aureus. The objects of this study were the inhibition zone diameter formed within the paper disk due to C. albicans and S. aureus. The method used in this study is an experimental method with a Completely Randomized Design (CRD) of six treatments (four isolates of endophytic fungi, antibiotics and negative control (aqueous sterile) and four replications. Data analysis using ANAVA with the F test. To see the effect between treatments used the Duncan test. The result showed that there was an influence of isolates endophytic fungi extracted from the leaves of the henna plant to inhibit the growth of C. albicans and S. aureus. Furthermore, the results of the Duncan test analysis revealed that there were no significant differences from each treatment on inhibiting the growth of C. albicans, while there were significant differences of each treatment on inhibiting the growth of S. aureus.

1. Introduction
Antimicrobes are active agents obtained from plants with healing effects which can inhibit or kill the growth of pathogenic microbes. Puspadewi, et al. stated that the bulbs of the Dayak onion (Eleutherine palmifolia (L.) Merr.) can be utilized as antimicrobe as it contains secondary metabolic compounds [1]. Natural antimicrobes are usually derived from secondary metabolic of certain plant extracts. One of the plants that can be extracted as natural antimicrobe is the leaves of the henna plant (Lawsonia inermis).

The leaves of the henna plant (L. inermis) is commonly used by people across Indonesia, including in Gorontalo province. The local name in Gorontalo for this plant is tilangge. The locals believe that tilangge can be used to heal the rotting wound that often found in fingers. The leaves of this plant are usually crushed and directly applied to the wound and then wrapped with a clean cloth. Simatupang described that candida albicans is a normal flora that usually found in the digestive tract, also the mucous membrane of the respiratory tract, vagina, skin, and under the finger’s nails of the hand and foot [2].
Further, Pratiwi described that S. aureus is a normal flora in human being especially found in the skin, nose, and mouth [3]. Extensive studies have been done to inhibit the growth of pathogenic microbe by using the henna plant (Lawsonia inermis) utilizing only the extract of this plant. However, overexploitation of the herbal plant to obtain its secondary metabolic compound can threaten the existence of this plant in nature. To reduce this, this study only uses the isolates of endophytic fungi which expected to produce the expected amount of secondary metabolic compounds without having an extraction to the plant directly. The purpose is to make the production of secondary metabolic compounds from the herbal plant efficient.

This study was to investigate the ability of endophytic fungi isolates from the henna plant (Lawsonia inermis) toward the growth of Candida albicans and Staphylococcus aureus.

2. Data and method

2.1. Research site and timeline
This study was conducted at the microbiology laboratory of the Biology department in the faculty of Mathematics and Natural Sciences of Universitas Negeri Gorontalo for four months from October 2017 to January 2018.

2.2. Research objects
The objects in this study were the growth of C. albicans and S. aureus which observed from the inhibition zone diameter formed within the paper disk.

2.3. Research method
This study was used the experimental method with utterly randomized design which consists of six treatments and four replications. Hence there were 24 units of test objects.

2.4. Equipment
The equipment used in this study are: laminar airflow, oven, incubator, autoclave, Erlenmeyer, test tube, measuring cup, petri dish, glass object, ose needle, micropipette, microscope, centrifuge, analytical balance, Bunsen, hot plate, spatula aluminum foil, glass stirrer, tweezers, calipers, scissors, cutter, tissue, and camera.

2.5. Ingredients
The ingredients used in this study were: the leaves from the henna tree (Lawsonia inermis), aqua, spiritus, NaOCl solution 1 % (Sodium Hypochlorite), alcohol 70 %, testing microbe C. albicans and S. aureus, paper disk, NA (Nutrient Agar) medium, PDA (Potato Dextrose Agar) medium and PDB (Potato Dextrose Broth) medium.

2.6. Working procedure
The working procedure in this study is as follow:

2.6.1. Equipment sterilization. The equipment made from glasses that would be used in this study such as petri dish, Erlenmeyer, test tube, measuring cup, and glass object were washed with antiseptic washing soap then dried. The dried equipment were wrapped with paper and aluminum foils and put into the oven with the temperature of 170°C for 1 hour. The Ose and tweezers were sterilized using the heat on the Bunsen fire.

2.6.2. Ingredients preparation. The leaves of henna tree were taken from the center of the stalk. It is about ten dark green colored leaves.
2.6.3. Creation of the growth media. The growth media that would be used were: NA, PDA, and PDB. The NA, PDA, and PDB media were created based on the packaging and number of a petri dish or test tube that would be used by weighing the NA, PDA, and PDB then dissolved with deionized water. The media were heated using the hot plate till boiled, then sterilized using autoclave in the temperature of 121 °C with 15 pounds pressure for 15 minutes. The sterilized media were then poured into petri dish aseptically and were let to be solid in the room temperature.

2.6.4. Isolation of endophytic fungi from the henna tree leaves (Lawsonia inermis). Before the endophytic fungi were isolates from its host, the henna leaves were sterilized. The selected henna leaves were the dark green-colored leaves. The leaves were washed using flowing water, then soaked with 70% of alcohol for 30 seconds to get rid of the oily layer on the surface of the leaves. Then, the leaves were soaked with the 1% of NaOCl solution for 5 minutes. The final preparation step for these leaves was to rinse the leaves three times with sterilized water for 1 minute to clean the leaves from the NaOCl solution. Then, the leaves were dried on top of the sterilized tissue. Further, the leaves were cut into small pieces of 4-6 mm wide and 1-2 cm length.

The pieces of the henna leave put into the solidified PDA (Potato Dextrosa Agar) medium on the petri dish where each dish was filled with five pieces of samples. This process was conducted in laminar airflow. Further, the samples were incubated for 2-14 days at the temperature of 25-27 °C (room temperature). The observation was carried out on day 3 to day 14. The isolates endophytic that showed the fungi morphology were transferred into the new PDA medium [4].

2.6.5. Purification of the isolate endophytic fungi. The purification was carried out to all fungus colonies that grew and considered different from the produced endophytic fungi based on the macroscopic morphology appearance which consists of the color and shape. Hasiani, et al. stated that each pure isolate was duplicated for culture stock and research culture [4].

2.6.6. Observation of the morphology of endophytic fungi. The isolates endophytic fungi were observed macroscopically and microscopically. For the macroscopic observation, the fungus colony shape, the edge shape, the surface and the color of colony were observed. Whereas for microscopic observation, the magnifying glass and the microscope were used.

2.6.7. The productivity of the secondary metabolic compounds. The supernatant that contains the secondary metabolic compounds can be produced through fermentation method. Fermented endophytic fungi were produced using the Potatoes Dextrose Broth (PDB) medium, which aimed to produce the extract that contains secondary metabolic compounds from the isolates endophytic fungi. Three parts of the pure colony of the isolates endophytic fungi in PDA petri dish that has been incubated for seven days were taken using a sterilized straw. The pieces of this fungi then inoculated into the 20 mL of liquid fermented PDB in 100 mL size of Erlenmeyer pumpkin. The pieces then statistically incubated in the room temperature of 29 °C for seven days [5]. Then the liquid medium from the fermented process were put into the centrifuge tube of 15 mL which has been previously sterilized, then the centrifuged were operated in the speed of 3000 rpm for 20 minutes. The supernatant produced from this centrifuge were filtered using the filter paper. This filtered supernatant was used to test the microbe activities as the testing liquid [6].

2.6.8. The microbe activity testing. The antimicrobial activity test toward the C. albicans and S.s aureus were carried out using the paper disk method, by dipping the paper disk into the supernatant of the fermented endophytic fungi. The paper disk which has absorbed the supernatant then placed into the medium that contains the C. albicans and S. aureus microbe, then incubated in the room temperature for two days.
2.7. Data collection method
The data were obtained by measuring the diameter of the inhibitory zone formed within the paper disk by using the calipers Davis and Stout stated that if the diameter of the inhibitory zone is less than 5 mm then the inhibitory activity was considered weak, if the inhibitory zone diameter is between 5-10 mm then the inhibitory activity is considered moderate, and if the inhibitory zone diameter is 10-19 mm then the inhibitory activity is considered strong, and if the inhibitory zone diameter is 20 mm or more than the inhibitory activity is considered very strong [7].

2.8. The technique of data analysis
The data obtained in this study then analyzed using the statistical application SPSS software. The data were analyzed using the variance analysis (ANOVA) with an F test to see whether there is an influence of isolates endophytic fungi on the growth of C. albicans and S. aureus. If the influence were found then the next test, the Duncan test was carried out to see the significant differences between each treatment.

3. Result and discussion

3.1. Isolates endophytic fungi from the henna leaves (Lawsonia inermis)
Based on the morphologic observation of the endophytic fungi colonies, there were four endophytic fungus isolates with different colonies characteristics as presented in Figure 1. Noverita mentioned that the endophytic fungus produced from the host tree could produce different types of isolates and a different number of variation [8]. This was an adaptation mechanism of the endophyte toward the micro ecology and physiological condition that specific to each host, hence, will influence the different composition of endophytic colonies composition and level of infection of the host plant by the endophytic fungus from a similar location.

3.2. Antimicrobial activity of the endophytic fungi isolates
Based on the results, the influence of the endophytic fungi isolates the growth of C. albicans and S.s aureus microbes. It reveals that the average obstacles zone diameter in the treatment is presented in Table 1.
Table 1. The average diameter of the obstacles zone (mm) of the endophytic fungi isolates toward the growth of *C. albicans* and *S. aureus*

| Endophyte fungus isolates from | the average diameter of obst zone (mm) | average diameter of obst zone (mm) |
|-------------------------------|--------------------------------------|-----------------------------------|
|                               | *C. albicans*                         | *S. aureus*                       |
| Treatment A (control -)       | 0                                    | 0                                 |
| Treatment B (Isolate Fungus A)| 7.83                                 | 8.13                              |
| Treatment C (Isolate Fungus B)| 7.64                                 | 12.44                             |
| Treatment D (Isolate Fungus C)| 7.68                                 | 9.23                              |
| Treatment E (Isolate Fungus D)| 7.86                                 | 11.29                             |
| Treatment F (Control +)       | 18.71                                | 24.39                             |

Table 1. showed that the average obstacles zone diameter for positive control group is larger than the treatment groups and the negative control group. Differences in average diameter of obstacles zone for *C. albicans* microbe and *S. aureus* were also shown in the table 1.

The averages of obstacles zone diameter for *C. albicans* in Isolate A, B, C, D, control positive and control negative were 7.83, 7.64, 7.68, 7.86, 18.71 and 0 mm, respectively. The averages of *S. aureus* obstacles zone diameter in isolate A, B, C, D, control positive and control negative were 8.13, 12.44, 9.23, 11.29, 24.39 and 0 mm, respectively. The best isolate of endophyte fungus to inhibit the growth of *C. albicans* was isolate D, while the best isolate to inhibit the growth of *S. aureus* was isolate B. The isolates from endophytic fungi was more effective in inhibiting the growth of *S. aureus* than inhibiting the growth of *C. albicans* (Figure 2).

The inhibition that formed in *C. albicans* made by each isolate of the endophytic fungi can be categorized to be only able to inhibit the growth of Candida albicans microbe, while in *S. aureus* the isolate was not only able to inhibit the growth of this microbe but also it is able to inhibit the growth of this *S. aureus* microbe. The results can be seen from the shape of the obstacles zone formed in each testing microbe. For *C. albicans* microbe, the formed obstacles zone was only partial for each isolate of the endophytic fungi, which means that the growth of the microbe can only be inhibited. Whereas in *S. aureus*, the formed obstacles zone for isolates A and C were only partial, which means that the isolates were only able to inhibit the growth of the microbe. Meanwhile, the isolates of the endophytic fungus B and D were able to inhibit the growth of the microbe, which means the isolates were able to eliminate the *S. aureus* microbe.

Based on the diameter of the obstacles zone or inhibitory zone formed in the testing microbe, the *C. albicans* and *S. aureus*, the diameter of the inhibitory zones was different. In *C. albicans*, the inhibitory zone of the isolates endophytic fungi was categorized as moderate, whereas *S. aureus* in the isolates A and B have a moderate inhibitory zone, while isolates B and C have a strong inhibitory zone.
The differences of the inhibitory zone for each testing microbe in each treatment can be seen in the histogram below.

**Figure 3.** Average diameter of an inhibitory zone formed in each treatment

### 3.3. Statistical analysis result

Based on the result of the F test it was obtained that the endophytic fungi isolate of the leaves of the henna tree (*Lawsonia inermis*) has influenced on the growth of *C. albicans* and *S. aureus* microbes. The result of hypothesis 1 test by using the variance analysis (ANAVA) with F test showed that the F count > F table or that the value of Sig < α: 0.05 hence. It could be concluded that the H₀ was rejected and H₁ was accepted.

Further analysis using the Duncan test showed that there was no significant difference for each treatment of endophytic fungi isolates from the leaves of the henna tree (*Lawsonia inermis*) in inhibiting the growth of *C. albicans* microbe. However, there was a significant difference in inhibiting and eliminating the growth of the *S. aureus*. The detail is presented in Table 2, where different notation was indicated significant differences in each treatment, whereas similar notation was indicated no significant differences in each treatment.

| Endophyte Fungus Isolate | Average diameter Obstacle zone (mm) | Average diameter Obstacles zone |
|--------------------------|-------------------------------------|--------------------------------|
|                          | *C. albicans*                      | *S. aureus*                    |
| Treatment A (control -)  | 0⁰                                 | 0⁰                             |
| Treatment B (Fungus Isolate A) | 7.83ᵇ                             | 8.13ᵇ                          |
| Treatment C (Fungus Isolate B) | 7.64ᵇ                             | 12.44ᶜ                         |
| Treatment D (Fungus Isolate C) | 7.68ᵇ                             | 9.23ᵈ                          |
| Treatment E (Fungus Isolate D) | 7.86ᵇ                             | 11.29ᵉ                         |
| Treatment F (Control +)  | 18.71ᶜ                            | 24.39ᶠ                         |

Each inhibitory or obstacles zone formed in *C. albicans* and *S. aureus* have different shape and diameter of the inhibitory zone. The small and significant difference in the inhibitory zone showed in this study indicated different sensitivity toward the *C. albicans* and *S. aureus*. Differences of activity of the endophytic fungus isolate in inhibiting the growth of the microorganisms which are more sensitive toward the *S. aureus* than the toward the *C. albicans*. This was because the *S. aureus* which was gram-positive bacteria with the simple cell wall.

Harti stated that the cell wall of the gram-positive bacteria was composed of one layer of peptidoglycan [9]. Peptidoglycan is the main component of the cell wall of the bacteria which rigid and
responsible for keeping the integrity of the cell and determining its shape. Meanwhile, the \textit{C. albicans} has six layers of cell walls, hence the possibility for antimicrobial compounds to fully inhibit its growth is small. Khomariah described that the wall of the \textit{C. albicans} which composed of six layers, the outer layer is called fibrillar layer, then mannoprotein layer, $\beta$-glucan, $\beta$-glucan-chitin, mannoprotein and plasma membrane \cite{10}. The wall of the cell consists of 80-90\% carbohydrate, protein 62\% and lipid 1-7\%. Carbohydrate includes the branched polymer glucose ($\beta$-glucans), non-branched polymer N-acetyl-D glucosamine (chitin) and polymer mannoprotein (mannan).

Inhibition of the \textit{C. albicans} and \textit{S. aureus} growth was suspected due to the ability of the endophytic fungi isolate to produce secondary metabolic compounds. Hasiani et al. stated that the extract of isolates endophytic fungi from the leaves of the henna tree (\textit{Lawsonia inermis}) was able to produce the secondary metabolic compounds which consist of flavonoid, tripteron, and steroid \cite{4}.

Flavonoid works as protein denaturation. Hence, this process is suspected to disrupt the formation of the cell, thus changes the composition of the protein components. Putri mentioned that flavonoid compounds are the derivative of the phenol compounds \cite{11}. The activity of the phenol compounds is by destroying the lipid membrane of the microbe plasma. The membrane of the plasma is semipermeable and works to control the transportation of various metabolite into and out of the cell. The disruption or the destruction in the membrane of the plasma can inhibit the ability of the plasma as osmosis inhibitor and eliminate several biosynthesis processes within the membrane.

In addition to flavonoid, there is also steroid compounds from the tripteron group whose function is similar in destroying the membrane of the microorganism cell. Hence, the growth of the \textit{C. albicans} and \textit{S. aureus} can be inhibited. The steroid is assumed to eliminate the protein of the bacteria and the fungus through non-specific interaction between the bacteria and the fungus. Sapara stated that the steroid inhibits the growth of the microbe by interacting with the permeable phospholipid membrane of the cell toward the lipophilic compounds \cite{12}. Hence, the integrity of the cell changes then become vulnerable and lysis.

4. Conclusion

Based on this study, the following things can be concluded:

- There was influence of endophytic fungi isolates from the leaves of the henna tree (\textit{Lawsonia inermis}) on inhibiting the growth of \textit{C. albicans} and eliminating the \textit{S. aureus}.
- There were no significant differences in each isolates endophytic fungi treatment in inhibiting the growth of the \textit{C. albicans}. However, the significant differences were found in the ability of the different isolates on eliminating and inhibiting the growth of the \textit{S. aureus}.

5. Recommendations

Several recommendations in this study are:

- Further research needs to be carried out to investigate the ability of the isolates endophytic fungi from the leaves of the henna tree (\textit{Lawsonia inermis}) in inhibiting the growth of other pathogenic microorganisms.
- Further research needs on the identification of endophytic fungus which is formed a symbiosis with the leaves of the henna tree (\textit{Lawsonia inermis}) needs to be done.
- Further research needs on the production of antibiotic from the isolate of endophytic fungus from the henna tree (\textit{Lawsonia inermis}) in inhibiting the growth of several pathogenic microorganisms.
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