Evaluation of the potency of endophytic fungi associated with *Artemisia annua* as antibacterial and antioxidant

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Abstract. *Artemisia annua* has been known as a traditional herbal medicine. The purpose of this study was to evaluate the potential of eight endophytic fungi associated with *A. annua* as antibacterial and antioxidant. Preliminary screening for antibacterial and antioxidant activities was carried out by the TLC-bioautographic method, and the active extracts were further analyzed for their MIC and IC₅₀ values by serial microdilution method at 96 microwell plates. The results showed that eight endophytic fungi were able to inhibit the growth of *Staphylococcus aureus* with MIC values ranging from 64 to > 256 µg/ml. In the meantime, five endophytic fungi inhibited the growth of *Eschericia coli* with MIC values of 256 µg/ml. One isolate, which has a very strong antibacterial activity against *S. aureus* (MIC = 64 µg/ml), was TdAaCb-2. Four endophytic fungi extracts were active as an antioxidant by acting as DPPH free radical scavengers. The results of this study indicated that the endophytic fungi extracts associated with *A. annua* were promising natural sources for antibacterial and antioxidant.

1. Introduction

Endophytic fungi are microorganisms that reside in the healthy tissue of the host plant without affecting adverse effects. The diversity of endophytic fungi is enormous, and they present in almost of plant species [1]. The presence of endophytic fungi can be beneficial for the host plant as plant growth, a defense mechanism against herbivores, pathogens, and insects [2].

Endophytic fungi have been known as potential sources of novel natural products [3]. Natural products from endophytic fungi have the potential to be applied in agriculture, medicine, and food industry [4]. Besides, many bioactive compounds have isolated from endophytic fungi with cytotoxic, insecticidal, antimicrobial, and anticancer activities in the last two decades [4]. Considering that endophytic fungi are potential sources in producing natural bioactive compounds, the investigation on the bioactivity of endophytic fungi isolated from traditional medicinal plants is very important [3].

*Artemisia* is a genus of small herb and shrub, which belongs to Asteraceae [5], comprises more than 500 species and distributed mainly in the temperate zones of North America, Europe, and Asia [6]. The genus *Artemisia* has an important role in ethnopharmacological medicine around the world [7]. Traditionally, several species of *Artemisia* have been used in the treatment of cough, cold, malaria, headache, diabetes [8], restoration of declining mental function, cardiac stimulant, and anthelmintic [9,10].
Several studies have been done on the bioactivity of endophytic fungi isolated from several *Artemisia* species. A previous study by Qian et al. [3] showed that endophytic fungi isolated from *A. argyi* exhibited antimicrobial and anti-tumor activity. *Colletotrichum* sp. isolated from the stem of *A. annua* is potential as an antibacterial against *Bacillus subtilis, Staphylococcus aureus, Sarcina lutea,* and *Pseudomonas* sp. with minimal inhibitory concentrations (MICs) ranging from 25 to 75 μg/ml [11]. Because of the enormous diversity of endophytes and their bioactivity potential, this study aims to isolate the endophytic fungi associated with *A. annua* from Cibodas Botanical Garden, West Java-Indonesia and evaluate their activity as antibacterial and antioxidant.

2. Materials and methods

2.1. Endophytic fungi isolation

Healthy plants of *A. annua* were collected from Cibodas Botanical Garden, West Java-Indonesia. The endophytic fungi associated with *A. annua* were isolated based on the surface sterilization method [12]. Plant materials were washed under tap water and surface sterilized by rinsing the plant material with 70% alcohol for two min, followed by 5.3% Na-hypochlorite for five min, 70% alcohol for 0.5 min, and sterile distilled water for one min. Plant materials were dried and cut aseptically to expose the interior surface to culture media. Three fragments of plant materials were placed onto the CMMA (Corn Meal Malt Agar) and incubated for one week at room temperature. After incubation, the emergence of fungi was isolated to obtain a single isolate. The hyphal tip of emergence fungi was transferred to Potato Dextrose Agar to obtain a pure culture.

2.2. Fungal identification

The fungal identification was carried out based on a morphological approach. Observation of both macroscopic and microscopic phenotypic characters was carried out for morphological identification. Macroscopic characterization included observation of color, texture, shape of the colony, drop of exudate, surface, and inverted color of fungal culture. For microscopic observation, fungal mycelia were placed in one drop of 1% blue lactophenol solution. Microscopic characterization was carried out under a light microscope by observing hyphae, clamp connections, hyphae pigmentation, spores, septate, and other reproductive structures [13].

2.3. Cultivation of endophytic fungi in broth media and extraction

The pure isolate of endophytic fungus was cultured in 200 ml Potato Dextrose Broth in 500 ml Culture flask and incubated for three weeks without agitation under dark condition. After incubation, biomass and culture media were macerated with ethyl acetate three times. The ethyl acetate layer was separated and concentrated by a rotary evaporator. The concentrated extract was subjected to antioxidant and antibacterial screening by the TLC-bioautographic method or stored at low temperatures for further use.

2.4. Antibacterial screening by TLC-bioautography

Ten microliters of endophytic fungi extract (10 µg/ml) were transferred onto the TLC silica plate (Merck F254). The antibacterial screening was carried out against *S. aureus* and *E. coli* by Dot-Blot test [12]. The active extracts were further analyzed to determine the active antibacterial compounds. After the extract was transferred, the TLC plate was developed with an eluent system of dichloromethane: methanol (10:1) to separate chemical compounds. Dried plates were dipped in the bacterial solution and incubated for 18 hours at 37°C under humid condition. After incubation, the plates were sprayed with INT (*p*-iodonitrotetrazolium) to visualize the growth inhibition.

2.5. Detection of antioxidant activity by TLC-DPPH

Ten microliters of endophytic fungi extract (10 µg/ml) were transferred onto the TLC silica plate (Merck F254). Screening of antioxidant activity of extracts was carried out by Dot-Blot assay and eluted TLC-plates. After the extract was transferred, TLC plates were sprayed with a solution of 0.2% 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. Extract with antioxidant activity was indicated by
yellowish color against the purple background. The antioxidant compounds were separated by the eluent systems of dichloromethane: methanol (10:1). The developed plates were dried to remove the solvent and sprayed with DPPH. The yellowish band indicated the antioxidant compounds.

2.6. Determination of Minimum Inhibitory Concentration (MIC)
The MIC value of extracts was carried out by serial microdilution using a 96-well microplate. Wells in the first row were filled with Mueller Hinton Broth (100 µl), extract (10 µl with the concentration of 10.24 mg/mL DMSO), and sterilized aquadest (90 µl and then mix thoroughly. The mixture of the 1st well of each vertical row was transferred (100 µl) to the 2nd well and mixed thoroughly. The serial dilution was continued to the 4th well. One hundred µl from the 4th well was removed and discarded. After finish diluting, each well was added with 100 µl bacterial suspension (10^6 CFU/ml) then incubated at 37ºC for 24 hours. Microdilution of each extract was carried out in triplicates. After incubated overnight, each well was added with 10 µl INT. Minimum Inhibitory Concentration (MIC) is the lowest concentration before the color changes [14].

2.7. Determination of IC_{50} value of the antioxidant activity
The first row of 96 well microplates was filled with 195 µl Methanol and 5 µl extract (10.24 mg/mL) and mixed thoroughly. The second well to the 4th well of each vertical row was filled with 100 µl Methanol. Subsequently, the mixture of the 1st well was transferred (100 µl) to the second well, mixed thoroughly. The serial dilution was continued to the 4th well. One hundred µl from the 4th well was removed and discarded. After finish diluting, each well was added with 100 µl DPPH solution (61.5 µg/ml). The plates were incubated for 90 minutes under dark conditions at room temperature. After incubation completed, the absorbance of extracts was measured at 517 nm using a microplate reader (Varioscan flash, Thermo scientific). The antioxidant activity of free radical scavenging activity (% inhibitory concentration) was calculated using the following formula [15]:

\[
IC(\%) = \frac{(A_o) - (A_s)}{(A_o)} \times 100\%
\]

Where: \(A_o\) is the absorbance of the control, \(A_s\) is the absorbance of extracts with varying concentrations. The IC_{50} (half inhibitory concentration) value was obtained from a linear equation of % inhibition and extract concentration. Antioxidant activity index (AAI) was calculated by the following equation [15]:

\[
AAI = \frac{The \ final \ concentration \ of \ DPPH \ in \ the \ reaction (\mu g/ml)}{IC_{50} (\mu g/ml)}
\]

3. Results and discussion

3.1. Thin layer chromatography
TLC is a qualitative method to separate secondary metabolites in the extract. This method has been widely used because it is simple, low cost, and rapid, and uses a small amount of sample [16]. Chromatogram of endophytic fungi associated with A. annua showed that several spots represented several chemical compounds with various Rf. Spraying the TLC plates with vanillin sulfate and cerium sulfate is to visualize separate chemical compounds in the extract. The color of chemical compounds represents different compounds, such as polyphenols, steroids, alkaloids, and flavonoids [17]. Chromatogram of endophytic fungi extracts associated with A. annua developed in dichloromethane-methanol (10:1 v/v) were performed in Figure 1.
3.2. Antibacterial screening by TLC-bioautography

Endophytic fungi extracts isolated from *A. annua* were subjected to TLC-Bioautography analysis to evaluate their antibacterial activity, which was carried out with or without a developing plate.

**Figure 1.** Chromatogram of endophytic fungi extracts associated with *A. annua* developed in dichloromethane-methanol (10:1 v/v), (a) viewed under 254 nm wavelength, (b) viewed under 366 nm wavelength, (c) sprayed with vanillin reagent, and (d) sprayed with cerium reagent. No.1-8 are the extracts corresponding to Table 1.

**Figure 2.** TLC dot-blot assay for antibacterial activity of endophytic fungi extracts associated with *A. annua* against (a) *S. aureus* and (b) *E. coli*. No.1-8 are the extracts corresponding to Table 1, C+: chloramphenicol.

**Figure 3.** TLC-bioautogram of antibacterial activity of endophytic fungi extracts associated with *A. annua* against (a) *S. aureus*, and (b) *E. coli* developed in dichloromethane-methanol (10:1 v/v). The number represents the extracts number corresponding to Table 1.
Several endophytic fungi extracts under investigation showed growth inhibition against \textit{S. aureus} and \textit{E. coli} (Figure 2). The formation of white spots around the extract and white bands indicated growth inhibition of bacteria. White spots or white bands indicated that no-reduction of INT to the colored formazan did not occur because of the presence of antibacterial compounds [18]. Figure 2 and 3 showed the differences in the sensitivity of endophytic extracts in inhibiting the growth of \textit{S. aureus} and \textit{E. coli}. It might be caused by differences in the membrane structure between \textit{S. aureus} (Gram-positive bacteria) and \textit{E. coli} (Gram-negative bacteria). Gram-negative bacteria have an outer membrane composed of hydrophobic polysaccharide chains as a barrier and protection of the inner membrane and cell wall, whereas Gram-positive bacteria lack this membrane [16].

3.3. Antioxidant screening by TLC-bioautography
Screening of antioxidant activity of endophytic fungi was performed by TLC-DPPH bioautography. TLC-chromatogram in Figure 4 and 5 showed yellowish spots or bands on the purple background.

![Figure 4](image1) ![Figure 5](image2)

**Figure 4.** TLC dot-blot assay for antioxidant activity of endophytic fungi extracts associated with \textit{A. annua}. No.1-8 are the extracts corresponding to Table 1, C+: (+)-Catechin.

**Figure 5.** TLC-bioautogram of antioxidant activity of endophytic fungi extracts associated with \textit{A. annua} developed in dichloromethane-methanol (10:1 v/v). The number represents the extracts number corresponding to Table 1.

Yellowish spots or bands indicated the capability of the extracts or compounds to donate hydrogen [19], so DPPH free radical reduced to yellow colored diphenyl-picyrylhydrazine [20]. Color intensity indicated the antioxidant capacity [21].

3.4. Determination of Minimum Inhibitory Concentration (MIC) of endophytic extract
Endophytic fungi exhibited antibacterial activity were further analyzed for their MIC value by microdilution. The results are shown in Table 1.

| No | Sample | MIC (µg/ml) | \textit{S. aureus} | \textit{E. coli} |
|----|--------|-------------|-------------------|-----------------|
| 1  | AkAaCb-1 | >256        | -                 |                 |
| 2  | AkAaCb-2 | >128        | 256P              |                 |
| 3  | AkAaCb-3 | >256        | -                 |                 |
| 4  | BtAaCb-1 | >256        | 256P              |                 |
| 5  | BtAaCb-2 | 256         | -                 |                 |
| 6  | DnAaCb-1 | >256        | >256              |                 |
| 7  | TdAaCb-1 | 256         | 256               |                 |
| 8  | TdAaCb-2 | 64          | 256               |                 |
| 9  | Chloramphenicol | 4      | 8                 |                 |
The MIC value of endophytic fungi against *S. aureus* ranged from 64->256 µg/ml, while MIC value against *E. coli* ranged from 256->256 µg/ml. The smaller the MIC value, the better the antibacterial activity. TdAaCb-2 has good antibacterial activity against *S. aureus* with the MIC value of <100 µg/ml [22]. These results showed that *S. aureus* was more sensitive than *E. coli*. This result is consistent with the result of TLC-bioautography. Antibacterial activity of the endophytic extract related to its chemical compounds.

3.5. Determination of IC\textsubscript{50} value of the endophytic extract

The IC\textsubscript{50} of endophytic fungi that exhibited antioxidant activity on TLC-bioautography was performed by microdilution on 96-well microplate. The results in Table 2 showed that only one extract (AkAaCb-3) exhibited strong antioxidant activity with an IC\textsubscript{50} of 16.45 µg/ml and antioxidant activity index (AAI) of 1.87 [15]. Antioxidant activity of the endophytic fungi was contributed by chemical compounds contained in the extract.

**Table 2.** The IC\textsubscript{50} values and antioxidant activity index (AAI) of endophytic fungi extracts associated with *A. annua*.

| No | Sample      | IC\textsubscript{50} (µg/ml) | AAI value | Fungal taxa based on morphology |
|----|-------------|-------------------------------|-----------|--------------------------------|
| 1  | AkAaCb-1   | 60.05                         | 0.51      | Coelomycetes                   |
| 2  | AkAaCb-2   | >128                          | < 0.24    | Hyphomycetes                   |
| 3  | AkAaCb-3   | 16.45                         | 1.87      | Coelomycetes                   |
| 4  | BtAaCb-1   | -                             | -         | Dematiaceae                    |
| 5  | BtAaCb-2   | -                             | -         | *Colletortrichium* sp.         |
| 6  | DnAaCb-1   | >128                          | < 0.24    | Hypomycetes                    |
| 7  | TdAaCb-1   | -                             | -         | *Colletortrichium* sp.         |
| 8  | TdAaCb-2   | -                             | -         | *Neopestalotiopsis* sp.        |

4. Conclusion

The results of this study suggest that endophytic fungi associated with *A. annua* may contain valuable bioactive compounds exhibiting antibacterial and antioxidant activity. The results of this study support the utilization of *A. annua* as traditional medicine. However, a further step of the study is still needed to isolate and characterize the bioactive compound. The isolation and chemical structure elucidation of the active compounds from the active extracts are under progress.

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