Antioxidant activity of endophytic fungi from young and old leaves of cinnamon plants from Bogor, Indonesia

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Abstract. In addition to the bark, the leaves of the cinnamon plant are a source of natural antioxidant compounds and inhabited by endophytic fungi. The use of endophytic fungi from cinnamon leaves as a source of antioxidant compounds is not much done. This study aimed to determine the antioxidant activity of endophytic fungi were isolated from young and old leaves of cinnamon plants. Isolation of endophytic fungi was done by planting leaf tissue on Potato Dextrose Agar media after surface sterilization with 70% ethanol for 1 minute, 5.3% sodium hypochlorite for 5 minutes, and 70% ethanol for 30 seconds, respectively. Antioxidant activity is carried out based on the α, α-diphenyl-β-picrylhydrazyl free radical scavenging method. The ethyl acetate extract of endophytic fungi with the highest antioxidant activity on each type of leaf was carried out by profiling chemical compounds using GCMS. The extracts of eight endophytic fungal isolates obtained had antioxidant activity. Cb.Dm3 and Cb.Dt2 endophytic fungal extracts had the highest antioxidant activity with IC50 values of 62.80 and 8.11 µg.ml−1, respectively. Profiling chemical compounds showed that both isolates contained antioxidant compounds with Cb.Dt2 isolate containing cinnamaldehyde. Cb.Dt2 endophytic fungal extract has the potential as a potential source of natural antioxidant compounds.

1. Introduction

Increased free radicals can increase the pathogenesis of several diseases in humans. Within normal limits, the body has a natural defense ability to cope with the increase in free radicals. Superoxide compounds can be suppressed by antioxidant compounds. Antioxidants are an interesting topic today because of their ability to protect the human body from attacks by several diseases caused by free radical reactions. The use of synthetic antioxidants to protect against damage from free radicals has been reported to cause dangerous side effects. Therefore, it is necessary to search for new sources of antioxidants that come from nature [1].

The search for chemical compounds that have the potential as antioxidants from nature continues. Medicinal plants are an alternative therapy because they are relatively safer compared to synthetic drugs. Some medicinal plants have been used for generations as traditional medicine and contain antioxidant compounds including cinnamon. The part of the cinnamon plant that is commonly used as traditional medicine is the bark. This is because cinnamon bark contains several active compounds as medicinal ingredients, especially compounds with antioxidant activity [2]. In addition to the bark,
cinnamon leaves also have high utilization potential. Traditionally, cinnamon leaves are commonly used as food [3]. In addition, cinnamon leaf extract contains antioxidant compounds [4].

The ability of a medicinal plant to cure a disease is closely related to the content of the chemical compounds it contains. The composition and content of active compounds in plants, such as cinnamaldehyde in cinnamon, are also still closely related to the presence of endophytic microbes in them. Endophytic fungi are one of the endophytic microbes that live in the tissues of the host plant and are widely studied about the content of active compounds including antioxidants. Endophytic fungal extracts from several parts of the cinnamon plant, including leaves, are reported to have antioxidant activity [5]. The ability of cinnamon leaf extract to produce antioxidant compounds is not accompanied by further information that reports the effect of differences in the ability of endophytic fungi on different parts of the leaf maturity level. Therefore, this study aims to determine the antioxidant activity through the method of free radical scavenging of endophytic fungal extracts from young and old leaves of cinnamon plants from Bogor.

2. Materials and Methods

2.1. Plant Materials

Plant material in the form of young and old cinnamon leaves (Cinnamomum burmannii) was obtained from the Gunung Mas Agritourism area, Bogor, West Java, Indonesia located at S 6°42’32’’ and E 106°58’4’’. The samples taken were twigs containing healthy old (green) and young (red) leaves. The area where the twig was cut has been sealed to prevent contamination. The plant samples were taken to the laboratory on the same day to immediately isolate the endophytic fungi. Herbarium plants are then made to be identified at the Bogoriense Herbarium, Research Center for Biology, LIPI.

2.2. Isolation of Endophytic Fungi

Leaf samples were picked from their branches and washed with running water for 10 minutes to clean from dust and then dried at room temperature using sterile filter paper. Young and old cinnamon leaves were then surface sterilized [6]. Surface sterilization was done by soaking young and old leaves of cinnamon plants in 70% ethanol for 1 minute, 5.3% sodium hypochlorite for 5 minutes, and 70% ethanol for 30 seconds. The sample was dried on sterile filter paper in laminar. The samples were then cut 1x1 cm long and then placed on 15 ml the Potato Dextrose Agar (PDA) media in a Petri dish (9 cm in diameter). Each repetition was done 3 times. The Petri dish was then incubated at room temperature. All fungal colonies that grow then purified by taking a small number of hyphae and planted on 15 ml Potato Dextrose Agar (PDA) media and incubated at room temperature for 48-72 hours. Pure and well-grown colonies were then planted on 5 ml PDA slant media with 3 replications by adding isolate code and stored at 4°C.

2.3. Fermentation and Extraction of Endophytic Fungi

Endophytic fungal isolates that were 7 days old were taken by means of a hole with a sterile hole 6 mm in diameter and taken as many as 2 pieces to ferment in 250 ml Erlenmeyer flask containing 100 ml of Potato Dextrose Broth (PDB) media for 14 days. Fermentation is carried out on a shaker at a speed of 120 rpm at room temperature. After 14 days of fermentation, the filtrate and biomass of endophytic fungi were separated by filtering using sterile filter paper in a vacuum Buchner funnel. The filtrate was directly extracted with ethyl acetate (1:1) 3 times in a separating funnel. The filtrate extract was concentrated using a vacuum rotary evaporator flask until a dry extract was obtained [7].

2.4. Antioxidant Activity Test

The antioxidant activity test was carried out using the DPPH free radical scavenging method [8] with modification at a wavelength of 517 nm. A total of 1 mg of each endophytic fungal extract was dissolved in 1 ml of pro-analysis methanol to obtain 1000 μg.ml⁻¹ stock solution concentration. A total of 19.71 mg of DPPH was dissolved in 50 ml of pro-analysis methanol to obtain 1 mM DPPH
concentration. A total 1 mg of vitamin C was dissolved in 10 ml of pro-analysis methanol to obtain 100 μg.ml\(^{-1}\) stock solution concentration. A total of 1, 2, 5, 10, and 20 μl of stock solution of endophytic fungal extract and 2, 6, 10, 14, and 18 μl of vitamin C stock solution were put into a 96 well plate. A total of 40 μl of DPPH solution was added in filled well and finally added with pro-analysis methanol up to 200 μl. So that, the final extract concentrations were 5, 10, 25, 50, and 100 μg.ml\(^{-1}\) and vitamin C of 1, 3, 5, 7, and 9 μg.ml\(^{-1}\). As a blank, 40 μl of DPPH solution was added with pro-analysis methanol up to 200 μl into the well. All samples of the test solution, comparison standards, and blank were incubated at 37 °C for 30 minutes. The absorption of all samples and blanks were then measured at a wavelength of 517 nm. The antioxidant activity was obtained using the equation below and IC\(_{50}\) value which is a number that shows the concentration of the test sample that is able to inhibit the oxidation process by 50% obtained by making a linear curve between the concentration of the test solution (x-axis) and % antioxidant activity (y-axis).

\[
\%\text{Inhibition} = \frac{A - B}{A} \times 100\%
\]

A is blank absorption
B is sample absorption

2.5. Profiling secondary metabolites from endophytic fungi from cinnamon leaves

The ethyl acetate extract of endophytic fungi from young and old leaves of cinnamon with the highest antioxidant activity was followed by analysis using GCMS. Each potential extract of endophytic fungi of young and old leaves of cinnamon plants was dissolved in ethyl acetate at a concentration of 100 μg.ml\(^{-1}\). The extract solution were then injected into the GC-MS Shimadzu GC2010/MS QP2010 with the RTX-5MS column (30m x 0.15 mm ID x 0.25 μm) using Electron Impact (EI) and Negative Chemical Ionization (NCI) for the ionization method under the following conditions [9].

| Column Oven Temp. | 60°C |
|-------------------|------|
| Injection Temperature | 200°C |
| Pressure | 9.2 psi |
| Total Flow | 54 ml.min\(^{-1}\) |
| Column Flow | 1.08 ml.min\(^{-1}\) |
| Linear Velocity | 37.8 cm.sec\(^{-1}\) |
| Purge Flow | 3 ml.min\(^{-1}\) |
| Oven Temp. Program | 60°C for 3 min |
| Oven Temp. Program | 10 min increased to 235°C |
| Oven Temp. Program | 235°C for 20 min |
| Ion Source Temp. | 250°C |
| Interface Temp. | 250°C |
| Gas Sources | Helium |

3. Results and Discussion

3.1. Isolation of Endophytic Fungi

The results of plant determination show that the plant used was cinnamon (Cinnamomum burmannii). Isolation of endophytic fungi from young leaves and old leaves obtained a total of 8 isolates, namely Ch.Dm1-4 (origin of young leaves) and Ch.Dt1-4 (origin of old leaves) (Figure 1). Classification of isolate codes based on host type (young and old leaves), emergence during the isolation process and macroscopic morphological characters include the shape of colony growth, surface and reverse colors, colony surface character, presence or absence of concentric ring and radial furrow, and the presence or absence of exudate drops. The isolates were then stored as stock in PDA media in a test tube.
3.2. Fermentation and Extraction of Endophytic Fungi

Figure 2 shows that the acquisition of endophytic fungi ethyl acetate extract from the old leaves of Cb.Dt2 cinnamon isolate was the highest, while the Cb.Dt3 isolate from old leaves had the lowest extract acquisition. Obtaining different extracts from each isolate can be caused by the different ability of each endophytic fungi to produce secondary metabolites that are released into the fermentation media as their environment. Therefore, secondary metabolites are obtained by extracting the filtrate as a growth medium for endophytic fungi [10]. In addition, the high acquisition of ethyl acetate extract of Cb.Dt2 endophytic fungi may also be caused by the high content of semi-polar and slightly non-polar compounds such as oil which is interested when extracting with ethyl acetate. Therefore, in addition to the content of secondary metabolites, the content of compounds such as oil can also affect the acquisition of endophytic fungi ethyl acetate extract [9].

Figure 2. Product yield from ethyl acetate extract of endophytic fungi from cinnamon leaves.

3.3. Antioxidant Activity Test

In the antioxidant activity test, all extracts at a concentration of 100 µg.ml⁻¹ showed the ability to reduce free radicals. Values of inhibition varied between 21.88-90.28% from as shown in Table 1. Extracts of endophytic fungi Cb.Dm3 and Cb.Dt2 were the two extracts with the highest inhibition in each leaf type compared to other extracts so that it continued to determination for IC₅₀ values. The IC₅₀
value of ethyl acetate extract of endophytic fungi Cb.Dt2 was lower compared to Cb.Dm3, but both were still greater than the comparative control of vitamin C as shown in Table 2.

The strength of antioxidant activity can be grouped into very active categories if they have IC50 <10 µg.ml−1, active if they have IC50 11-100 µg/ml−1, and are inactive if they have IC50 > 100 µg.ml−1 [11]. From the test results, it can be seen that the antioxidant activity of Cb.Dm3 endophytic fungi ethyl acetate extract is in the active category because IC50 value is 11-100 µg.ml−1. Whereas the Cb.Dt2 extract was included in the very active category with the IC50 value <10 µg.ml−1 and approaching the positive control value was vitamin C which had an IC50 value of 3.06 µg.ml−1. Antioxidant activity of Cb.Dm3 and Cb.Dt2 endophytic fungal extract was better compared with Fusarium sp. isolated from cinnamon leaves (Cinnamomum loureiroi) which had an IC50 value of 98.04 µg.ml−1 [12].

Table 1. Screening of antioxidant activity of endophytic fungal extracts from cinnamon leaves. Cb.Dm1-Cb.Dm4 from young leaves and Cb.Dt1-Cb.Dt4 from old leaves.

| No. | Sample  | Antioxidant activity (%) ±SD |
|-----|---------|-------------------------------|
| 1   | Cb.Dm1  | 38.61±0.10                   |
| 2   | Cb.Dm2  | 36.64±0.03                   |
| 3   | Cb.Dm3  | 42.91±0.03                   |
| 4   | Cb.Dm4  | 21.88±0.02                   |
| 5   | Cb.Dt1  | 63.30±0.05                   |
| 6   | Cb.Dt2  | 90.28±0.07                   |
| 7   | Cb.Dt3  | 32.40±0.02                   |
| 8   | Cb.Dt4  | 54.59±0.04                   |

DPPH free radical scavenging method is a method commonly used in antioxidant testing in vitro. The principle of this method is the interaction of antioxidant compounds with DPPH free radicals which cause a change in purple DPPH compounds to yellow α, α-diphenyl-β-picrylhydrazine compounds which are more stable [13]. These changes are due to the ability of antioxidant compounds to donate hydrogen to free radical compounds [14].

Table 2. Antioxidant activity of endophytic fungal extracts from young and old leaves of cinnamon plants with the highest inhibition.

| No. | Sample | IC50 values (µg.ml−1) ±SD | Category |
|-----|--------|---------------------------|----------|
| 1   | Cb.Dm3 | 62.80±0.12                 | Active   |
| 2   | Cb.Dt2 | 8.11±0.36                  | Very active |
| 3   | Vit. C | 3.06±0.03                  | Very active |

3.4. Profiling secondary metabolites from endophytic fungi from cinnamon leaves

Profiling secondary metabolites based on NIST14 library from Cb.Dm3 endophytic fungi from young leaves of cinnamon can be seen in Table 3 and Figure 3, while for Cb.Dt2 isolates from old leaves can be seen in Table 4 and Figure 4. The data displayed in both tables are compounds that have a similarity index value of at least 90%. Several compounds have been detected from the ethyl acetate extract of endophytic fungi Cb.Dm3 and Cb.Dt2. Some of these compounds have been reported to have antioxidant activity. This is what causes both endophytic fungal extracts to have in vitro free radical scavenging activity.
Figure 3. GCMS chromatogram results of ethyl acetate extract of Cb.Dm3 endophytic fungi from young leaves of cinnamon.

The compounds contained in the endophytic fungi Cb.Dm3 ethyl acetate extract which have been previously reported to have antioxidant activity are propanoic acid ethyl ester and hexadecanoic acid methyl ester. The hexadecanoic acid methyl ester group or another name palmitic acid has also been reported to have antioxidant activity [15]. This compound is a derivative of ascorbic acid (vitamin C) in the form of water-soluble fats. Propanoic acid ethyl ester, or also called propionic acid ethyl ester is a compound that has antioxidant activity [16].

The compounds contained in the endophytic fungi Cb.Dt2 ethyl acetate extract which have been previously reported to have antioxidant activity are n-hexadecanoic acid, 3-eicosene (E)-, and Cinnamaldehyde (E)-. The compound n-hexadecanoic acid or known as methyl palmitate compound, which is found in many vegetable oils, has been reported to have the antioxidant ability [17]. The 3-eicosene compound found in cyanobacterium Nostoc muscorum is reported to have antioxidant activity [18]. Cinnamaldehyde is the dominant compound found in cinnamon plants which have been known to have strong antioxidant activity [19].

The antioxidant activity of cinnamon leaves is strongly influenced by environmental conditions and the plant itself, one of which is the level of leaf maturity. Similarly, the antioxidant activity of the extracts of endophytic fungi that are symbiotic with the leaves of cinnamon plants is also influenced by this. This can be seen in the antioxidant activity of Cb.Dt2 endophytic fungal extract from old leaves is higher than the Cb.Dm3 endophytic fungal extract from young leaves. This is in line with previous studies that reported that the more mature the age of cinnamon leaves, the higher the antioxidant activity [20]. This was also reinforced from the GCMS results that the compounds detected in both extracts were higher in endophytic fungi extracts from old leaves.
Table 3. Chemical composition of endophytic fungi Cb.Dm3 ethyl acetate extract from young leaves of cinnamon based on GCMS analysis.

| No. | R. Time | Area (%) | Compounds                                               | MW  | CAS#    | Fragmentation |
|-----|---------|----------|---------------------------------------------------------|-----|---------|---------------|
| 1   | 2.058   | 15.95    | Isopropyl acetate                                       | 102 | 108-21-4|               |
| 2   | 2.268   | 65.41    | Butane, 1-ethoxy-                                       | 102 | 628-81-9|               |
| 3   | 2.395   | 0.30     | Propanoic acid, ethyl ester                             | 102 | 105-37-3|               |
| 4   | 2.467   | 0.73     | 2-Ethoxypentane                                         | 116 | 1817-89-6|               |
| 5   | 3.286   | 0.29     | Cyclopentane, 1-ethyl-3-methyl-, cis-                    | 112 | 2613-66-3|               |
| 6   | 18.380  | 0.51     | Phenol, 4-(1,1-dimethylpropyl)-                          | 164 | 80-46-6 |               |
| 7   | 18.660  | 0.51     | n-Pentadecanol                                          | 228 | 629-76-5|               |
| 8   | 20.125  | 0.92     | Hexadecanoic acid, methyl ester                         | 270 | 112-39-0|               |
| 9   | 20.651  | 0.33     | 1,2-Benzene dicarboxylic acid, butyl 2-ethylhexyl ester | 334 | 85-69-8 |               |
| 10  | 20.813  | 0.95     | 1-Nonadecene                                            | 266 | 18435-45-5|              |
Table 4. Chemical composition of endophytic fungi Cb.Dt2 ethyl acetate extract from old leaves of cinnamon based on GCMS analysis.

| No. | R. Time | Area (%) | Compounds                          | MW  | CAS#       | Fragmentation |
|-----|---------|----------|------------------------------------|-----|------------|---------------|
| 1   | 3.176   | 9.24     | Cyclohexane, 1,3-dimethyl-         | 112 | 591-21-9  |               |
| 2   | 3.304   | 3.27     | Cyclopentane, 1-ethyl-3-methyl-    | 112 | 3726-47-4 |               |
| 3   | 3.357   | 4.55     | Cyclopentane, 1-ethyl-2-methyl-, cis- | 112 | 930-89-2 |               |
| 4   | 3.467   | 2.67     | Cyclohexane, 1,2-dimethyl-, trans- | 112 | 583-57-3 |               |
| 5   | 10.654  | 7.56     | 1H-Indene, 1-methylene-            | 128 | 2471-84-3 |               |
| 6   | 12.039  | 8.81     | Cinnamaldehyde, (E)-              | 132 | 14371-10-9 |               |
| 7   | 18.667  | 7.39     | 3-Eicosene, (E)-                  | 280 | 74685-33-9 |               |
| 8   | 20.132  | 8.89     | Hexadecanoic acid, 15-methyl-, methyl ester | 284 | 6929-04-0 |               |
| 9   | 20.819  | 9.79     | 1-Nonadecene                       | 266 | 18435-45-5 |               |
Figure 4. GCMS chromatogram results of ethyl acetate extract of Cb.Dt2 endophytic fungi from the old leaves of cinnamon.

The IC50 values indicate that both extracts have strong and very strong antioxidant activity categories. For endophytic fungi Cb.Dt2 extract is even close to vitamin C activity as a positive control. This is possible closely related to the number of antioxidant compounds contained in it and the composition of antioxidant compounds. GCMS results show that ethyl acetate extract of Cb.Dt2 endophytic fungi contain cinnamaldehyde compounds which have been known as compounds with strong antioxidant activity and are a marker compound in cinnamon plants [19]. The presence of cinnamaldehyde compounds, which are abundant in the cinnamon plant, in the ethyl acetate extract of endophytic fungi from cinnamon leaves shows a close relationship between host plants and endophytic fungi. The ability of endophytic fungi to produce the same compounds as their host plant was first reported in 1993 [21] who stated that the endophytic fungi *Taxomyces andreanae* isolated from the *Taxus brevifolia* plant was able to produce taxol compounds which were also produced by the host plant. Several hypotheses about the ability of endophytic fungi to produce the same compounds as their host plants are due to the horizontal gene transfer process from plants to endophytic fungi [22] or the result of a co-evolutionary process [23]. In addition, the presence of cinnamaldehyde compounds in endophytic fungal extracts proves that endophytic fungi are capable of producing compounds that are similar or even identical to their host plants.

4. Conclusion
Eight endophytic fungal isolates have been found isolated from the young and old leaves of cinnamon plants which all have antioxidant activity through the mechanism of DPPH free radical scavenging. Ethyl acetate extract of endophytic fungi Cb.Dt2 derived from old leaves of cinnamon plants has the highest antioxidant activity and contains cinnamaldehyde active compound which is a marker compound on cinnamon plants.

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