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Antioxidant and Antimicrobial Activity of Endophytic Fungi Isolated from *Syzygium aqueum* Leaves

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Abstract. *Syzygium aqueum* is widely used medicinal plant species with ethnomedicinal values. Despite the plant, the endophytes are recognized as a rich source of secondary metabolites with potentially useful pharmacological properties. This study was aimed to identify the endophytic fungi from *S. aqueum* leaves and discover their antioxidant and antimicrobial activity. The ethyl acetate extracts of the isolated endophytic fungi were investigated for their antioxidant and antimicrobial activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and paper disc diffusion assay, respectively. The antimicrobial activity was evaluated against three pathogen bacteria: *Staphylococcus aureus*, *Salmonella thypi*, and *Escherichia coli*, and one yeast: *Candida albicans*. Three endophytic fungi were obtained from *S. aqueum* leaves. Based on morphological characteristics, those fungi identified as *Cochliobolus* sp. (D22), *Penicillium* sp. (D32), and *Fusarium* sp. (D41). *Penicillium* sp. (code D32) showed significant antioxidant potential activity as an also significant antimicrobial activity with IC₅₀ 59,16% and highest zone inhibition against *E. coli* 13.38 ± 0.25 mm. It is worth considering a further investigation of their bioactive secondary metabolites and isolates the bioactive compound for the antioxidant and antimicrobial agents.

Keyword: *Syzygium aqueum*, endophytic fungi, antioxidant, antimicrobial

4 Introduction

Oxidative stress is generally considered the starting point for the onset of several diseases (i.e., tissues, chronic inflammation, cancer) [1]. This oxidative stress could contribute to selecting resistant bacterial strains since reactive oxygen species (ROS) are revealed to be an essential driving force [2,3]. The need for new therapeutic strategies in targeting antioxidant pathways and new antibacterial agents can fight chronic infections [4]. Plant secondary metabolites are commonly used as alternative natural sources of antioxidants, which have industrial usage as food ingredients and dietary supplements [5,6].



Many previous reports indicate that plant extracts have rich antimicrobial and antioxidant properties [7,8]. One of the potential medicinal plants is *Syzygium aqueum*.

Syzygium aqueum is a native plant in Indonesia and Malaysia that belongs to the Myrtaceae family. In Indonesia, *S. aqueum* is well known as water guava. *S. aqueum* have been used as traditional medicine [9]. The leaves have been extensively used to treat, such as antibiotics, anti-inflammation, antioxidant, antityrosinase [10,11,12]. The efficacy of a plant as traditional medicine is related to the bioactive compounds contained in the plant extract. Currently, there is a growing interest to use natural bioactive compounds not just from plant extracts, but also from endophytic fungi [13].

Endophytes fungi live inside the host plant and participate in many biological processes without causing disease or other adverse effects. Endophytic fungi are one of the plant - symbionts that benefit their host's defense from natural enemies [14]. Endophytes fungi are known to be an important resource of various classes of secondary metabolites. Those secondary metabolites can be the same or different from their hosts, and this is very important to increase the adaptability of endophytic fungi and the host plants, such as tolerance to biotic and abiotic stresses [15, 16, 17]. The present study aimed to identify the endophytic fungi from *S. aqueum* leaves and discover their antioxidant and antimicrobial activity.

2. Material and Methods

2.1. Collecting Plant Sample

S. aqueum leaves obtained from Oganllir Regency, South Sumatra, Indonesia. The samples taken were fresh, healthy, and dark green leaves of the 6-9th sequence. The leaves samples were taken using the hand-picking method, and then they were brought to the laboratory for cleaning and sterilization.

2.2. Isolation of Endophytic Fungi

Leaves of *S. aqueum* were washed with running tap water and then air-dried. Sample fragments were successively surface sterilized by immersing each sample in 70% alcohol for ± 1 minutes, then immersed in 3% (w / v) sodium hypochloride (NaOCl) for 1 minute. After rinsing in sterile distilled water for ± 1 minute. The leaves are cut aseptically by 2 x 2 cm. The surface-sterilized fragments were placed in Petri dishes containing PDA supplemented with chloramphenicol (0.2 g/L) and then incubated at 30 ± 2 °C for seven days under dark condition. The fungal growth on leaves segments, the plates were monitored every day. Individual hyphal tips were transferred onto fresh PDA and incubated at 30°C for seven days. The pure cultures were numbered and maintained in PDA slants and kept at 4°C [18].

2.3. Identification of Endophytic Fungi

Macroscopic and microscopic features carried out the characterization of endophytic fungal isolates morphology. Macroscopic characterization is based on the colony growth pattern, texture, marginal, color, and other features. The microscopic characterization using the slide culture method. The endophytic fungal cultures were put onto slides, then added one drop of lactophenol blue reagent. Furthermore, these slides were examined using a light microscope. The morphological characteristics data are then compared with key identification books for fungi [19, 20].

2.4. Extraction and Cultivation

Each endophytic fungal isolate was cultivated. The inoculum fungi ($\pm 10^6$ spores/ml) were inoculated as much as 5% (v / v) into 300 ml PDB medium placed in 1-liter bottles and incubated at room temperature for 4-8 weeks. The color change indicates that secondary metabolite compounds have been formed. As a comparison, 300 ml of PDB media has been provided during cultivation, which is put into a 1-liter bottle. The medium containing secondary metabolites is partitioned in ethyl acetate and evaporated. Concentrated extract facts to the next stage of antioxidant activity to active extracts [20].

2.5. Antimicrobial activity

Antimicrobial activity was investigated using a paper disc diffusion assay. Each sample was dissolved in dimethyl sulfoxide 10% (DMSO; Merck, Germany). The antibacterial activity was evaluated against one gram-positive bacteria: *Staphylococcus aureus*, then two gram-negative bacteria: *Salmonella thypi* and *Escherichia coli*, then one yeast: *Candida albican*. Organisms were maintained on Muller-Hinton agar (MHA) (Tanaka, 1992). The resulting suspension's turbidity was diluted with sterile aquadest to match with a 0.5 McFarland turbidity standard (1 Macfarland is equivalent to approximately 3.0×10^8 CFU/mL). The extract concentration used for this assay was 1000; 500; 250 and 125 $\mu\text{g}/\text{mL}$. Absorbent disks (Watmann^o 3.5mm of diameter) were impregnated with 10 μl of the solution then placed on the surface of inoculated plates (90mm). Positive control discs of Ciprofloxacin 10 $\mu\text{l}/\text{ml}$ were excluded from the assay. Diameters of growth inhibition zone were measured after incubation at 37 $^{\circ}$ C for 24h. All experiments were performed in triplicate.

2.6. Antioxidant activity

Antioxidant activity was determined by using the DPPH method. Fraction obtained from extraction procedure was dissolved to make 1000; 500; 250; 125; 62.5; 31.25 and 15.625 $\mu\text{g}/\text{mL}$ concentration. 0.2 mL of each concentration was added by 3.8 mL DPPH 0.5 mM. As a standard antioxidant ascorbic acid was used, and at least three replicates of each concentration were considered. The mixture was homogenized and left in the dark tube for 30 minutes. The absorption was measured using a UV-Vis spectrophotometer at λ_{max} 517 nm. In this test, ascorbic acid was used as a standard positive control and methanol as a negative control. Antioxidant activity can represent by the detention value of DPPH absorption, which was calculated through absorption inhibiting percentage of DPPH and IC50 value [17, 21, 22].

$$\% \text{Inhibition} = \frac{\text{Control Abs.} - \text{sample Abs.}}{\text{Control Abs.}} \times 100$$

3. Result and Discussion

3.1. Endophytic Fungi of *Syzygium aqueum* leaves

Hyphae's appearance around leaf segments indicated the growth of endophytic fungi (EF) isolates. Fungal colonies that showed different morphological properties (shape, color, and size) were further purified. Three endophytic fungi isolates have been isolated from the leaves of *S. aqueum*, as shown in Figure 1 with isolate code D22, D32, and D41. Mycelia from isolated fungi were then observed macroscopically and microscopically on Potato Dextrose Agar (PDA) medium. Furthermore, this morphology was matched with the fungi identification book [e.g., in 19, 20].

EF isolate with code D22 shown in Figure 1A;1D. The colony has a cotton-like surface with white color and a dark reverse color. Microscopically, the hyphae with septic form and conidiophores upright, brown, branched, straight or curved, porous, subellipsoidal, mostly 4-cell, darker brown at 2-cell center, larger middle at peripheral cells, with indistinct hilar at the base. Characteristics of the isolates code D22 compared with the literature [19] closest to *Cochliobolus* sp.

Macroscopic feature of isolate code D32 (shown in Figure 1B) have spread out colony with dark green and white edges. The reverse colony is yellowish white. Microscopically shown in Figure 1E have upright hyaline conidiophores, branched with 2-3 metulas, each metula contains filial verticillate, each filial dangle conidia, conidia round green. Isolates code D22 compared with fungi key identification book [19] approaching to *Penicillium* sp

The isolate EF code D41 close identified as *Fusarium* sp. The colony is white with a more yellowish back color, cotton-like surface with a flowering pattern (Figure 1C). Conidiophores are erect, branched, elongate primary and secondary branches, carrying spore masses in the filial branches, with stalk and terminal vesicles: cylindrical vesicles. Phialosporous conidia, cylindrical (Figure 1F).

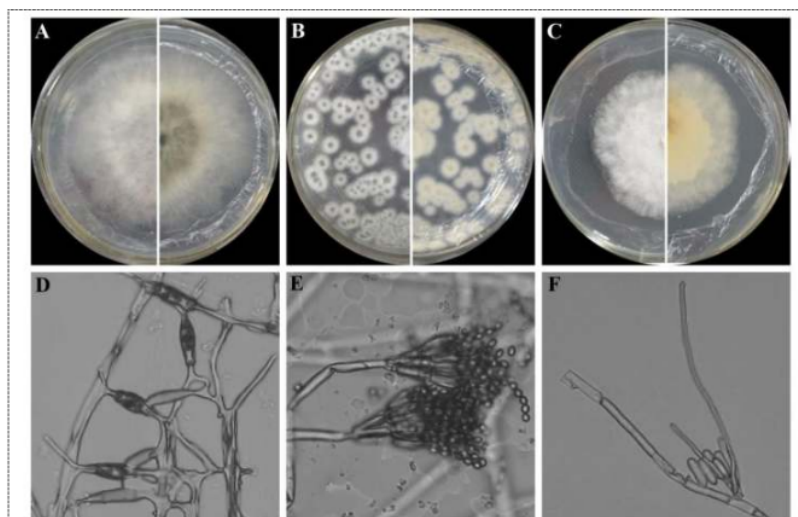


Figure 1. Morphology of Endophytic Fungi Isolates from *S. aqueum* Leaves. (A-C) Macroscopic morphology (D-F), microscopic morphology of isolate D22, D32 and D41 respectively.

3.2. Antibacterial Activity of Endophytic Fungi Extract

The antimicrobial activity of the endophytic fungi from *S. aqueum* leaves was assessed using the paper disc agar diffusion method by measuring the diameter of growth inhibition zones (IZ) at different concentrations level. The results antimicrobial activity are presented in Table 1. These results showed that the endophytic fungi extract had inhibition effect on the growth of three bacterial species, but low effect on yeast species (*C. albican*) with average IZ > 2 mm.

The antimicrobial activity corresponds to the concentration level of the extract, where the mean zone of inhibition decreases when the concentration is low from 1000 to 125 µg/ml (Table 1). *Penicillium* sp (code D32) have strong inhibition to *E. coli* and *S. thypi* with IZ 13,38 mm and 10,33 mm, but moderate inhibition against *S. aureus* with IZ 8,33 mm. The inhibition zone shows with clear zone arounds paper disc (Figure 2). *Fusarium* sp (code 41) also have strong antibacterial activity against *S. aureus* (IZ 11,27 mm) and *E. coli* (IZ 12,63 mm). The lowest antimicrobial activity shows in *Cochliobolus* sp. (Code 22).

Table 1. Antimicrobial Activity of Endophytic Fungi from *S. aqueum* Leaves

Samples	Concentration (µg/ml)	Growth Inhibition Zone diameter ^a (mm)			
		<i>E.coli</i>	<i>S. aureus</i>	<i>S.thypi</i>	<i>C.albican</i>
D22	1000	3.67 ± 0.15	4.57 ± 0.21	3.43 ± 0.06	1.80 ± 0.20
	500	5.37 ± 0.15	2.40 ± 0.10	3.10 ± 0.20	1.12 ± 0.06
	250	1.67 ± 0.38	2.07 ± 0.21	2.67 ± 0.06	0.70 ± 0.10
	125	1.37 ± 0.15	1.63 ± 0.15	2.43 ± 0.06	0.87 ± 0.12
D32	1000	13.38 ± 0.25	8.33 ± 0.15	10.33 ± 0.32	1.83 ± 0.06
	500	9.47 ± 0.15	7.83 ± 0.35	9.37 ± 0.21	0.77 ± 0.06
	250	5.30 ± 0.10	3.77 ± 0.06	5.03 ± 0.21	0.33 ± 0.06

D41	125	2.93 ± 0.32	2.10 ± 0.10	2.87 ± 0.15	0.23 ± 0.06
	1000	12.63 ± 0.55	11.27 ± 0.40	4.77 ± 0.61	1.23 ± 0.06
	500	6.60 ± 0.26	6.10 ± 0.46	4.97 ± 0.31	1.00 ± 0.10
	250	5.27 ± 0.83	5.53 ± 0.06	6.70 ± 0.44	1.33 ± 0.06
	125	0.90 ± 0.36	4.27 ± 0.45	3.63 ± 0.45	0.30 ± 0.10
Ciprofloxacin	30	31.37 ± 0.51	21.17 ± 0.15	30.17 ± 2.07	0.00 ± 0.00

^a Inhibition zone exclude the diameter of paper disc (6 mm)

Potential antimicrobial from *S. aqueum* leaves was reported to be able to inhibit the growth of clinical isolates such as *Staphylococcus aureus*, *Shigella dysenteriae*, *Escherichia coli*, *Salmonella thypi*, and *Vibrio cholerae* [23]. The in vitro antibacterial activity of several classes of polyphenols can be caused by direct action against bacteria, viruses and fungi, as well as suppression of microbial virulence factors [24].

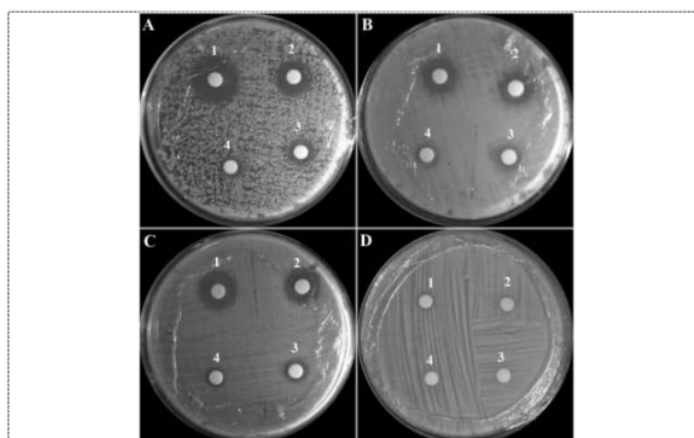
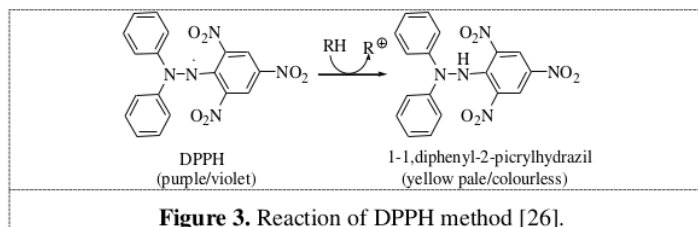


Figure 2. Inhibition zone of *Penicillium* sp (isolate code D32) against *E. coli* (A), *S. aureus* (B), *S. thypi* (C) and *C. albicans* (D) with concentration 1000; 500; 250 and 125 µg/ml (shown in number 1; 2; 3; and 4 respectively)

3.3. Antioxidant Activity of Endophytic Fungi from *S. aqueum* Leaves

Three endophytic Fungi were isolated from *S. aqueum* leaves and were labeled as D22, D32 and D41. Each of them had been extracted with ethyl acetate after the fungi isolating compounds from the *S. aqueum* leaves extract. Three fungi extracts were tested for antioxidant activity with DPPH method, which the result of reduction DPPH radicals cause discoloration from purple to yellow pale colour/colourless and the absorbance was measured with UV-Vis spectrophotometer at 515 nm [25]. The interaction of antioxidant compound with DPPH based on statement [25] is transfer of electron or hydrogen atom to DPPH radical and convert it to 1-1,diphenyl-2-picrylhydrazil (Figure 3). The IC50 value is used as a parameter to measure antioxidant activity in this research which the IC50 value of ethyl acetate of endophytic fungi is displayed in Table 2 .



These potential of *S. aqueum* supported by the bioactive compound. Phenolic and flavonoid compounds are the dominant compounds in the *S. aqueum* plant which have strong antioxidant activity [27, 28, 29]. The 5,7-dihydroxy, 6,8-dimethylflavone (demethoximatikinol) compounds isolated from the leaves of *S. aqueum* are known to have antioxidant activity [30].

Table 2. IC₅₀ values of ethyl acetate extracts of endophytic fungi isolated from *S. aqueum* leaves on antioxidant activity test using DPPH method.

Test sample	IC ₅₀ (µg/mL)
Ethyl acetate D22	168.14
Ethyl acetate D32	59.16
Ethyl acetate D41	384.05
Ascorbic acid ^a	22.23

IC₅₀ values at Table 2. Confirm that the extract endophytic fungi in ethyl acetate D32 have lowest value than other extract fungi. Fadhillah *et al.* [31] said that the lower IC₅₀ value corresponds with a higher antioxidant ability. From that statement we conclude that D32 is the greatest activity antioxidant and more active to scavenging free radical than others. Endophytic ethyl acetate D32 have a great activity because they have more secondary metabolites compound with many hydroxyl group such as polyphenols and flavonoid which they can be hydrogen-donating property of their hydroxyl group [32]. The 5,7-dihydroxy, 6,8-dimethylflavone (demethoximatikinol) compounds isolated from the leaves of *S. aqueum* are known to have antioxidant activity [30].

4. Conclusion

This study has shown that endophytic fungi isolated from *S. aqueum* leaves obtained isolate and identified as *Cochliobolus* sp. (D22), *Penicillium* sp. (D32) and *Fusarium* sp. (D41). All endophytic fungi have antioxidant and antimicrobial activity. *Penicillium* sp. (code D32) showed significant antioxidant potential activity as also significant antimicrobial activity with IC₅₀ 59,16% and highest zone inhibition against *E. coli* 13.38 ± 0.25 mm. It is worth considering further investigation of their bioactive secondary metabolites and isolate the bioactive compound for antioxidant and antimicrobial agent.

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