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ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF DARK PIGMENT EXTRACTED FROM *PHOMOPSIS* SP., AN ENDOPHYTIC FUNGI OF *ANNONA MURICATA*

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Abstract

Fungi may produce melanin, a polymeric pigment that protect fungi from unfavourable biotic and abiotic environment. This study aimed to evaluate potent of melanin of *Phomopsis* sp., an endophytic fungi of *Annona muricata* for its antioxidant and antimicrobial activities. Dark pigment on hyphae of *Phomopsis* sp that is expected as melanin was extracted using alkaline procedure. UV-visible spectra of pigment were like that of fungal and synthetic melanin. Antioxidant activity was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) method while antimicrobe activity was performed using MTT (Thiazolyl Blue Tetrazolium Blue) assay. The pigment extract had antioxidant activity with Antioxidant Activity Index (AAI) of 0.1104. The extract of 512 µg/ml decreased cell viability of bacteria *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium smegmatis*. However, the Minimum Concentration Inhibition (MIC) of those bacteria could not be observed until the concentration of 2048 µg/ml. Cell viability of yeast *Saccharomyces cerevisiae* was inhibited by concentration of 1024 µg/ml. The extract of 2048 µg/ml did not affect cell viability of *Candida tropicalis*. In conclusion, dark pigment of *Phomopsis* sp. had moderate activity of antioxidant and some bacteria and yeast. Further analysis would be needed to verify if the pigment is melanin.

Keywords: *Phomopsis* sp., *melanin, antioxidant, anti-microorganism*

Introduction

Phomopsis sp. is an endophytic fungi isolated from leaf of *Annona muricata* which has anti breast cancer activity [1]. When this endophytic fungi is cultured in Yeast Malt Broth or Yeast Malt A, dark pigment come out in the mycelia after several days of incubation at room temperature (figure 1). In a strain of *Phomopsis* sp., [2] concluded that dark pigment in the fungal mycelia is melanin based on solubility in solvents, UV-VIS, and infrared red spectrum.

Melanin are darkly pigmented polymers that protect organisms against environmental stress [3]. In fungi, the production of melanin significantly enhances the virulence of many important human pathogenic fungi and to the ability of fungi to survive in diverse hostile environments, capacity to alter cytokine responses, decrease phagocytosis, reduce the toxicity of microbicidal peptides, reactive oxygen species, and antifungal drugs as well as to play a significant role in fungal cell wall mechanical strength [4].

The properties of fungal melanin allow it to have diverse applications such as pharmaceutical, medical device, or antimicrobial. Melanin involves protection from radiation, given that fungal melanin is known for its radio absorptive properties, reducing oxidative stress, and enhancing survival in liver cells exposed to high doses of xenobiotic compounds provoking reactive oxygen species (ROS), for removing various toxins from a polluted environment, to facilitate the absorption of heavy metals, absorption of nuclear pollution, and the food packaging [5].

By using a method for fungal melanin extraction, we studied the dark pigment of *Phomopsis* sp. for its antimicrobial and antioxidant activities. This study aimed to investigate the possibility of melanin content of *Phomopsis* sp. that have activity against some microorganism and of antioxidant. We hope that the results might reflect the role of the pigment in protection fungi from unfavourable environmental conditions. From industrial application point of view, the data contribute the possibility of *Phomopsis* sp. as source of melanin.

Methods

Materials

Microbial Cultures

Bacteria *E. coli* InaCC-B4, *S. aureus* InaCC-B4, mycobacterium *M. smegmatis* NBRC 3082, yeast *Candida tropicalis* and *Saccharomyces cerevisiae* were obtained from Indonesian Culture Collection (InaCC) LIPI, Indonesia. Endophytic fungi *Phomopsis* sp. (InaCC F1033) was used for dark pigment source.

Procedure

Growth conditions

The bacterial and yeast cultures were grown on 10 ml Nutrient Broth, NB (HIMEDIA) or Yeast Malt Broth, YMB (HIMEDIA) in 50 ml Erlenmeyer flask, and incubated on a shaker with 100 rpm at room temperature for 3 days. *Phomopsis* sp. was grown on 300 ml NB in 5000 ml Erlenmeyer flask, incubated on a shaker with 100 rpm at room temperature for 30 days.

Extraction of pigment

Extraction of pigment was conducted according to melanin extraction procedure [2] [6]. A total of 5.733 grams of dry dark-pigmented mycelia dissolved with 10 ml of 1 N KOH autoclaved for 20 minutes at 121°C, then cooled and filtered with filter paper. The filtrate was then precipitated with 4 N HCl. Extraction was continued by centrifugation for 20 minutes at a speed of 5000 g. The pellets were taken and then dried in an oven at 70 °C. The dried extract was washed using chloroform, methanol, and ethyl acetate (1:1:1) to remove other secondary metabolites possibly toxic towards microorganisms, then dried. Melanin that has been washed and dried was dissolved in DMSO for assay antimicrobial activity.

UV-Visible spectroscopic analysis of pigment

UV-Visible Spectroscopic analysis was carried out according to [2].

A small amount of pigment was dissolved in 1M KOH, and the absorption (200 to 600 nm) was read in a double beam spectrophotometer. The spectrum was recorded as the absorption spectrum of alkaline

extract of Phomopsis pigment using 1 M KOH as the reference blank

An example. In this example we can see that there are footnotes after each author name and only the antioxidant activity assay is based on the measurement of the scavenging capacity of samples towards DPPH (α, α-diphenyl-β-Picrylhydrazyl) [7] [8].

Antioxidant activity.

A serial of concentrations of methanol-dissolved samples of 100 µl was prepared in 96-well microplate. Each well then added with 100 µl DPPH (61.50 µg/ml). Methanol was used as negative control, while epigallocatechin gallate (EGCG) was used as positive control. The microplate was incubated in the dark at room temperature for 90 minutes. The absorbance of samples was measured at 517 nm. The linear regression equation was then constructed to determine concentration of sample scavenge half of DPPH free radical (IC50). The Antioxidant Activity Index was calculated by calculated by dividing final concentration of DPPH (30.75 µg/ml) by IC50 (µg/ml) according to [7].

Determination of Minimum Inhibition Concentration (MIC)

Antimicrobial activities were determined as Minimum Inhibition Concentration (MIC) using MTT (Thiazolyl Blue Tetrazolium Blue) according to [9] [10] [11]. Extracts were dissolved in DMSO and water. Aliquots of 50 µl of NB (bacteria and mycobacteria) or YMB (yeast) containing series of extracts concentrations were added to each well of a 96-well microplate. The concentrations ranged from 1 µg/ml to 2024 µg/ml. Cell suspension (50 µl) was added to the appropriate wells (1% v/v). Plates were incubated at room temperature for 1 (bacteria and yeast) or 3 days (mycobacterium. Thereafter, 10 µl (bacteria and mycobacterium) or 20 µl of the MTT.

Results

Extraction of pigment

We obtained 0.377 g dried pigment from 5.733 g pigmented mycelia that was dissolved in 1 M KOH and precipitated with 4 N HCl. Since melanin of this fungi has never been analyzed, we are not sure that this pigment is melanin. However, procedure for melanin extraction applied towards these fungi successfully produced dark pigment. The UV / Visible spectrum of the pigment, extracted from these fungi showed strong absorbance in the UV region. The absorbance maxima were in the about of 250 nm and progressively decreased in the visible wavelengths.

Antioxidant activity of pigment.

Antioxidant activity (AAI) was principally measured based on amount of antioxidant molecule (melanin) needed to react with half of DPPH, a stable free radical (IC50, the half- maximal inhibitory concentration). The index of antioxidant activity is then defined by comparing of initial concentration of DPPH in the reaction mixture and IC50. We compared AAI of pigment with EGCG which is a strong antioxidant. AAI of *Phomopsis* sp. pigment was 0.1104 whereas Epigallocatechin gallate was 12.443. Activity of antioxidant of the pigment was moderate.

Antimicrobial activity of pigment.

Antimicrobial activity is observed by measuring cell viability of microbes treated with antimicrobial agents. Viable cells reduce MTT which is yellow to formazan (purple). The colour of death cells will be therefore yellow. MIC of *Phomopsis* sp. pigment against *E. coli*, *S. aureus*, and *M. smegmatis*, yeast *C. tropicalis* and was yeast *S. cerevisiae* was >512 µg/ml, >512 µg/ml; >512 µg/ml; >2048 µg/ml, and 2048 µg/ml respectively. We compared antibacterial and antifungal activities of the pigment with those of antibiotics and antifungal drug. Antibacterial or antifungal activity of the pigment was lower than antibiotic or antifungal drug respectively. MIC of streptomycin against *E. coli*, and *S. aureus*, was ≤ 16 and ≤ 2 µg/ml respectively. MIC of rifampicin against *M. smegmatis* was ≤ 8 µg/ml. MIC of nystatin against yeast *C. tropicalis* and *S. cerevisiae* was > 256 µg/ml and ≤ 256 µg/ml respectively.

Discussion

Procedure for melanin extraction applied towards these fungi successfully produced dark pigment. This research is the first time of study on black pigment content of *Phomopsis* which has not been analyzed for melanin content. However, the UV /

Visible spectrum of the pigment, extracted from these fungi showed strong absorbance in the UV region (figure 2). The absorbance maxima were in the about of 250 nm and progressively decreased in the visible wavelengths. This absorbance pattern is the typical absorption profile of synthetic melanin and that of extracted from a strain of *Phomopsis* sp. and *Pseudomonas balearica* [2] [6]. We suggested therefore, that the pigment we extracted from *Phomopsis* could be melanin. Although, further structural and chemical analysis should be done to clarify this hypothesis.

We can see that activity of antioxidant of pigment is not so strong compared to epigallocatechin gallate (table 1). Antioxidant Activity Index (AAI) of pigment is 0.1104. According to AAI established by Scherer and Godoy [7], AAI of pigment is moderate while epigallocatechin gallate is strong. Though antioxidant activity is moderate, the role of pigment in protecting fungi from oxidative stress caused by unfavourable environment could not be neglected. Pacelli et al [12] demonstrated that melanin is effective in protecting fast and slow growing fungi from various types of ionizing radiation. Photoprotection property of this fungal melanin allow the fungi attenuate light intensity which produce ROS. ROS then partially react with melanin. In this case, antioxidant activity of melanin may be due to photoprotection rather than free radical scavenger activity, however it researches on melanin role in cells is needed to answer this question. The ability of photoprotection of this pigment is then needed to study. It is also important to note that we observed the activity of extracted pigment which is not connected to living cells. Extracted melanin could be structurally deferent with its origin in the living cell [13] [14] and as consequence antioxidant property of melanin in living cell could be underestimated.

Regarding antimicrobial activity of the pigment, the data is presented in table 2. At concentration of 512 µg/ml, pigment decrease viability of *E. coli, S. aureus, and M. smegmatis* but the MIC value was not observed until the concentration of 2048 µg/ml. Compared to the MIC value of relevant antibiotic (Streptomycin and Rifampicin), the activity of weak. The MIC of pigment against yeast *S. cerevisiae* and C. tropicalis was 2048 µg/ml and >2048 µg/ml respectively. Compared to the MIC value of relevant antibiotic (Nystatin), this activity is low. The mechanism toxicity pf the fungal pigment is not known. However, it is known that antioxidant compound due to free radical scavenging activity, in certain circumstance may have adverse effect (prooxidant). Antioxidant compound EGCG, depend on its microenvironment, can be a prooxidant as well [15] [16]. As a prooxidant, EGCG is toxic towards microorganism. Melanin which has antioxidant activity may have also prooxidant activity in a certain condition. Recently, melanin extracted from horsehair was reported as prooxidant against bacteria. It contains a high degree of redox active catechol groups, which can produce ROS. This finding suggests that melanin pigments may serve as agents with unique redox chemistry and ROS generation capability [17].

Production of dark pigment in endophytic fungi *Phomopsis* grown outside its living host (*A. muricata*) may be a beneficial condition protecting the fungi from unfavourable environment. Since *Phomopsis* is a culturable fungi, it is interesting to take advantage of it for producing biomaterial in the field of health, pharmacy, and bioremediation. This research revealed that Endophytic fungi *Phomopsis* which produced dark pigment that is similar in UV-visible spectra to fungal and synthetic melanin. The pigment had antioxidant and antimicrobial activities. Further research is needed to reveal potential of *Phomopsis* sp. as source of melanin that could be used in bioindustry in the field of health, pharmacy, and bioremediation.

Conclusion

The pigment extract had antioxidant activity with Antioxidant Activity Index (AAI) of 0.1104. The extract of 512 µg/ml decreased cell viability of bacteria Escherichia coli, Staphylococcus aureus, Mycobacterium smegmatis. However, the Minimum Concentration Inhibition (MIC) of those bacteria could not be observed until the concentration of 2048 µg/ml. Cell viability of yeast Saccharomyces cerevisiae was inhibited by concentration of 1024 µg/ml. The extract of 2048 µg/ml did not affect cell viability of Candida tropicalis. In conclusion, dark pigment of Phomopsis sp. had moderate activity of antioxidant and some bacteria and yeast. Further

analysis would be needed to verify if the pigment is melanin.

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Figure 1 Phomopsis sp. grown on YMB media

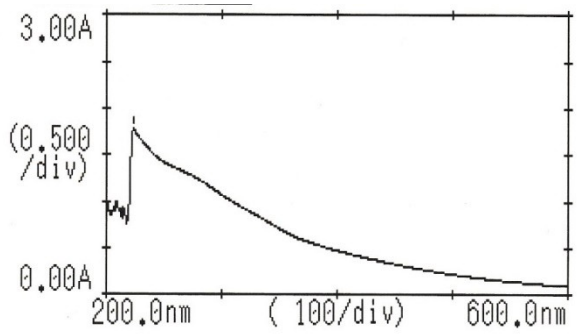

Figure 2 UV-visible spectra of dark pigment of *Phomopsis* sp.

Table 1 Antioxidant activity of pigment extracted from *Phomopsis* sp.

Sample	Antioxidant Activity Index (AAI)
Phomopsis sp pigment	0.1104
Epigallocatechin gallate	12.443

Table 2 Antimicrobial activity of pigment extracted from *Phomopsis* sp.

Sample	Targeted Microorganism	Minimum Inhibition Concentration (MIC, µg/ml)
Phomopsis sp. pigment	*E. coli*	>512
Streptomycin	*E. coli*	≤ 16
Phomopsis sp. pigment	*S. aureus*	>512
Streptomycin	*S. aureus*	≤ 2
Phomopsis sp. pigment	*M. smegmatis*	>512
Rifampicin	*M. smegmatis*	≤ 8
Phomopsis sp. pigment	*C. tropicalis*	>2048
Nystatin	*C. tropicalis*	> 256
Phomopsis sp. pigment	*S. cerevisiae*	2048
Nystatin	*S. cerevisiae*	≤ 256