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Antifungal activity of lignans isolated from *Phyllanthus myrtifolius* Moon. against *Fusarium oxysporum*

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ABSTRACT

Seven lignan compounds (**1**-**7**) were isolated from the leaves of *Phyllanthus myrtifolius* Moon. and were evaluated for their *in vitro* antifungal activities against an opportunistic filamentous fungus, *Fusarium oxysporum*, using the Clinical and Laboratory Standard Institute (CLSI) (M38-A2) standard method. The lignans phyllamyricin C (**1**), retrojusticidin B (**2**), phyllamyricin A (**3**), phyllamyricin F (**4**), justicidin B (**5**), phyllamyricin E (**6**) and piscatorin (**7**) were found to be active against *F. oxysporum*. Their minimum inhibitory concentration (MIC) values were in the range of 4.0-32.0 μg mL^{-1} and minimum fungicidal concentration (MFC) values were in the range of 8.0-62.5 μg mL^{-1}. The isolated lignans also have inhibitory activity on the conidial germination of *F. oxysporum*. The results strongly suggest that lignans isolated from the leaves of *P. myrtifolius* can be developed as natural antifungal agents.

KEYWORDS: antifungal, *Fusarium oxysporum*, germination inhibition, lignan, *Phyllanthus myrtifolius*

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INTRODUCTION

Fusarium oxysporum is widely distributed in nature and is one of the most important fungal pathogens found in agricultural area, infecting a range of host plants [1]. The diseases include wilt, blight, little leaf, vascular wilt, corm and root rots, and damping-off. Among them, wilting of egg plants is one of the important diseases that cause significant reduction in agricultural production, and under favorable conditions can lead to a 100% loss. A number of serious infections caused by this species can be extremely difficult to manage, because this soil borne pathogen can persist for many years in the soil without a host [2]. Recently, the genus of *Fusarium* has also been reported to infect human cells (fusariosis). *F. oxysporum* species causes a broad spectrum of infections, including superficial, locally invasive, and disseminated infections. Although not many incidences of human infections caused by *Fusarium* have been reported, *F. oxysporum* has accounted for 20% cases of fusariosis, which are mostly onychomycosis [3].

Fusarium control is very important and can be achieved by means of synthetic fungicides. However, these synthetic fungicides can be harmful to the environment [4]. In addition, the development of resistance of pathogenic fungi towards the synthetic fungicides is also one of the increasing concerns.

Moreover, public awareness on these factors has increased interest in finding alternative protectants that are safer and more degradable and can replace the synthetic fungicides. Therefore, there is a growing interest to explore the possibility of using natural compounds as anifungals [5].

The plants of the genus *Phyllanthus* (Euphorbiaceae) are widely distributed in most tropical and subtropical countries, and have long been used in folk medicines [6]. Several compounds including alkaloids, flavonoids, lignans, phenols, and terpenes have been isolated from these plants. With reference to antifungal properties, some extracts such as those from *P. acidus*, *P. amarus*, *P. emblica*, *P. muellerianus*, *P. niruri*, *P. piscotarum* and *P. wightianus* show activities against several pathogenic fungi [7-13]. However, only a few antifungal compounds isolated from *Phyllanthus* have been reported. One of them is justicidin B, a lignan from *P. piscotarum* that inhibits the growth of three species of pathogenic fungi at a concentration range of 1-12 µg mL^{-1} [14]. This study reports the susceptibility test of seven lignans isolated from *P. myrtifolius* Moon. against an opportunistic fungi *F. oxysporum*.

MATERIALS AND METHODS

Plant materials

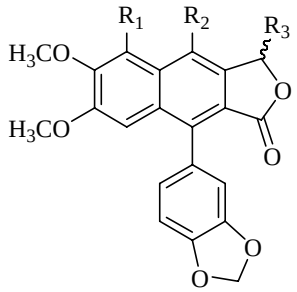
The plants were collected from Bogor, Indonesia. The voucher specimens (*P. myrtifolius*) were identified and deposited at the Herbarium Bandungense, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, Indonesia under specimen number HBG-12180.

Lignan isolation

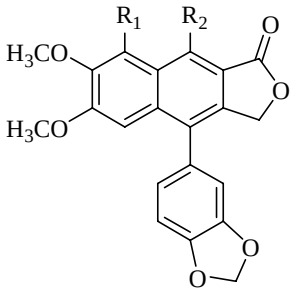
The dried and powdered leaves of *P. myrtifolius* (1.5 kg) were macerated with 2 L of methanol for 3 × 24 hours at room temperature to give a dark green methanolic extract (150.0 g). The methanolic extract was then re-extracted using acetone to obtain the acetone extract (65.0 g). A part of the extract (20.0 g) was fractionated by vacuum liquid chromatography (VLC) method, eluted by *n*-hexane-EtOAc of increasing polarity (9:1, 8:2, 7:3, 6:4, 1:1, 3:7, 2:8) to give seven fractions (F1-F7). Purification of the fraction F3 (143.0 mg) was performed by radial chromatography (eluent *n*-hexane-EtOAc = 8:2) to obtain phyllamyricin A (**3**) (16 mg). Simplification of the fraction F4 (751.0 mg) with radial chromatography gave retrojustisidin B (**2**) (20.0 mg) and justisidin B (**5**) (15.0 mg), while purification of the fraction F5 (518.0 mg) by the same method using *n*-hexane-diisopropyl ether (8:2) yielded phyllamyricins C (**1**) (14.0 mg), E (**4**) (10.0 mg), F (**6**) (11.0 mg), and piscatorin (**7**) (28.0 mg). All molecular structures, shown in Figure 1, were elucidated using ^{1}HNMR and ^{13}CNMR data (Agilent 500 MHz DD2 system), and by direct comparison with previously reported data [14-16].

Preparation of antifungal agents

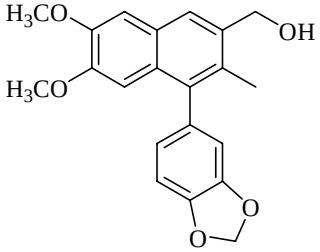
Each isolated lignan and amphotericin B, purchased from Sigma Chemical Co. (St. Louis, MO, USA),



Phyllamyricin C (**1**), $R_1 = OCH_3$, $R_2 = R_3 = H$
Phyllamyricin E (**4**), $R_1 = R_2 = H$, $R_3 = OCH_3$
Justicin B (**5**), $R_1 = R_2 = R_3 = H$
Piscatorin (**7**), $R_1 = R_2 = H$, $R_3 = OH$



Retrojusticidin B (**2**), $R_1 = R_2 = H$
Phyllamyricin A (**3**), $R_1 = OCH_3$, $R_2 = H$



Phyllamyricin F (**6**)

Figure 1. Structures of isolated lignans from *P. myrtifolius*.

were dissolved in 10% dimethylsufoxide (DMSO) in water. A 10% of DMSO did not affect the growth of *F. oxysporum* [17]. The concentration of lignans and amphotericin B were standardized to 1.0 mg mL^{-1} or 0.1%, and were stored at 4 ^{o}C prior to use.

Conidia preparation

F. oxysporum ATCC 44187 was obtained from the American Type Culture Collection (Rockville, MD). The strain was cultured and maintained on potato dextrose broth (PDB) (Difco, Spark, MD, USA) and PDB supplemented with 1.5% of microbial agar (Difco, Spark, MD, USA) at 35 ^{o}C for 7 days. Conidia suspensions of *F. oxysporum* were prepared by the method of CLSI M38-A2 [17]. In brief, the seven-day-old colonies were covered with approximately 1.0 mL of sterile 0.85% phosphate buffer saline (PBS, Sigma-P3813) medium, and the suspensions were made by gently probing the colonies with the tip of a Pasteur pipette. The resulting mixtures of conidia and hyphal fragments were withdrawn and transferred into a sterile tube. After heavy particles were allowed to settle for 3 to 5 min, the upper homogenous suspension was collected and mixed with a vortex mixer for 15 s to give a conidial suspension free from heavy particles. Conidia quantification was done by plating 0.05 mL of a 1:100 diluted conidial suspension on PDA plates and were incubated at 35 °C for 48 h. Based on the number of fungal colonies observed after the incubation, the conidial suspension was then made up to approximately 4.8×10^4 cfu mL^{-1} and was stored at 4 ^{o}C as a stock conidial inoculum.

Antifungal bioassay

Susceptibility test

Susceptibility test of the isolated lignans was carried out by a well plate-diffusion method. In brief, seven-days-old cultures of *F. oxysporum* were transfered using a sterile Pasteur pipette into the wells on the potato dextrose agar (PDA) plates. A 30 µL of each antifungal agent was dropped on the culture-contained well. The cultures were then incubated at 35 ^{o}C for 72 h. The growth of mycelia was observed daily, and the diameter of mycelia growth was measured. A negative control containing an aqueous 10% DMSO only and a positive control containing amphotericin B were included in each experiment. The percentage of mycelia growth inhibition was estimated by using the formula:

$$\% \text{ of inhibition} = 1 - \frac{\text{Diameter of treated colony}}{\text{Diameter of negative control colony}} \times 100\%$$

This experiment was done two times independently, each with a duplicate (n = 2 × 2).

***In vitro* susceptibility tests**

The minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of the isolated lignans from *P. myrtifolius* were determined according to broth microdilution method recommended by CLSI, approved standard M38-A2 [18]. In brief, a 100 µL of conidial inoculum of approximately 4.8×10^3 cfu mL^{-1} was transferred into each wells of a sterile disposable 96-well round bottom microtiter plate. Then, a two-fold dilution of antifungal agent (using 100 µL of 0.1% solution) was performed starting from well-12 (concentration of 500 µg mL^{-1}) till well-3 (concentration of approximately 1 µg mL^{-1}) and the remaining 100 µL from well-3 was discarded. Well-1 served as a negative control (only medium) and well-2 as a growth control (medium containing conidial inoculum). Microtiter plates were incubated aerobically at 35 °C for 48-72 hours. MIC is defined as the lowest concentration of antifungal agent that result in a complete inhibition of visible growth. MFC was determined by sub-culturing the suspension (10 µL) from each of the wells on PDA plates. The PDA plates were then incubated at 35 °C for 48 hours or until growth was seen in the growth control. MFC is defined as the lowest concentration of antimicrobial agents at which no growth occur in the PDA plate. This experiment was done two times independently, each with a duplicate (n = 2 × 2).

Conidial germination inhibition assay

The conidial germination inhibition assay was performed in PBS medium, according to previously reported method with slight modifications [17]. In brief, the stock inoculum suspension of 4.8×10^4 cfu mL^{-1} was diluted 1:10 in PBS medium to give a final concentration of 4.8×10^3 cfu mL^{-1}. Each concentration of lignans was diluted 1:10 in PBS medium containing 4.8×10^3 cfu mL^{-1}. The final concentrations of lignans were 0 × MIC, 0.5 × MIC, MIC, 2 × MIC, and 4 × MIC for each *F. oxysporum* isolate. Cultures (1 mL final volume) were incubated at 35 °C under 200 rpm agitation. The number of conidia was determined by plating 50 µL of each culture onto the PDA plate using sterile hockey stick-shaped spreader. The plates were incubated at 35 °C for 48 hours or until growth was seen in the negative control (0 x MIC) (72 h), and the percent inhibition of germination (Gi) was calculated using the formula given below [17]. This experiment was carried out two times independently, each with a duplicate (n = 2 × 2).

$$\%\ \mathrm{Gi} = \frac{(\text{Average germination (\%)control}) - (\text{Average germination (\%)treatment})}{\text{Average germination (\%)control}} \times 100\%$$

RESULTS AND DISCUSSION

The genus *Fusarium* contains important mycotoxin-producing species that cause fusariosis in plants, animals, and humans [1]. *Fusarium* such as *F. oxysporum* has become an increasingly common cause of breakthrough infections in immunosuppressed patients [3]. Thus, finding new compounds with strong antifungal activity is important in combating fusariosis. In the present study, seven lignans were isolated from the leaf extracts of *P. myrtifolius* (Figure 1) and were tested against an opportunistic filamentous fungus *F. oxysporum*. The antifungal activities of the lignans on *F. oxysporum* are summarized in Table 1.

The results showed that all isolated lignans were active against *F. oxysporum* with the inhibition zone ranging from 31 to 68% at concentrations of 0.1% (1000 µg mL^{-1}). The strongest inhibition was shown by compounds **3-6** (phyllamyricin A, phyllamyricin E, justicidin B and phyllamyricin F, respectively), which were able to give 62-68% of inhibition. Interestingly, the fungal inhibition of compounds **3-6** was higher than that of amphotericin B, a commercial antifungal agent, at the same concentration. Compounds **1-7** (phyllamyricin C, retrojusticidin B, phyllamyricin A, phyllamyricin E, justicidin B, phyllamyricin F, and piscatorin, respectively) inhibited the growth of *F. oxysporum* and had MIC values in the range of 4-32 µg mL^{-1}. In general, these MIC values were higher than that of amphotericin B (MIC 2.0 µg mL^{-1}). These results are comparable to previously reported data showing that antifungal agents isolated from plants are mostly milder compared to the commercial antifungal agent [19]. Moreover, these seven lignans **1-7** can completely kill the conidia of *F. oxysporum* and had MFC values of 62.5, 32.0, 62.5, 32.0, 8.0, 62.5, 32.0 µg mL^{-1}, respectively. Among the seven compounds, compound **1** (phyllamyricin C) shows the strongest antifungal activity with MIC and MFC values of 4.0 and 62.5 µg mL^{-1}, respectively.

The effect of each compound on conidia germination of *F. oxysporum* was tested at concentration of 0 × MIC to 4 × MIC for 48 or 72 h at 35 ^oC. The results are presented in Table 2. The lignans inhibit the germination of *F. oxysporum* in a concentration dependent manner. Compounds **4** and **7** (phyllamyricin E and piscatorin, respectively) completely inhibited the germination at concentration of 2 × MIC, while compounds **3** (phyllamyricin A) and **6 (**phyllamyricin F) gave the same effect at concentration 4 × MIC. These results are in agreement with the MFC values of these compounds (Table 1). However, compounds **1** (phyllamyricin C), **2** (retrojusticidin B) and **5** (justicidin B) at concentration of 4 × MIC did not completely inhibit the germination. The results obtained for compound **1** (phyllamyricin C) can be understood because of the fact that there was a substantial difference between their MIC and MFC values (MIC 4 µg mL^{-1}; MFC 62.5 µg mL^{-1} = 16 × MIC) (Table 1). However, the effect of compounds **2** (retrojusticidin B) and **5** (justicidin B) on the germination cannot be related to their MIC and MFC values. In general, the results show that the lignans isolated from *P. myrtifolius* can inhibit conidia germination of *F. oxysporum*, and to the best of our knowledge, this is the first report on the inhibition of conidia germination of *F. oxysporum* by lignans isolated from *P. myrtifolius.*

Table 1. Inhibition zone results, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of lignan compounds isolated from *P. myrtifolius* against *F. oxysporum* ATCC 44187.

Lignans	**Average inhibition zone (%) (1000 µg mL^{-1})**	**MIC (µg mL^{-1})**	**MFC (µg mL^{-1})**
Phyllamyricin C (**1**)	46	4.0	62.5
Retrojusticidin B (**2**)	48	16.0	32.0
Phyllamyricin A (**3**)	67	32.0	62.5
Phyllamyricin E (**4**)	68	16.0	32.0
Justicidin B (**5**)	67	8.0	8.0
Phyllamyricin F (**6**)	62	32.0	62.5
Piscatorin (**7**)	31	16.0	32.0
AMP-B (positive control)	45	2.0	2.0
DMSO (10%) (negative control)	NI	> 500.0	> 500.0

AMP-B = amphotericin B; NI = no inhibition.

Table 2. Effect of lignan compounds isolated from *P. myrtifolius* on conidial germination of *F. oxysporum* ATCC 44187 after 48 or 72 h.

Lignans	**Concentration (MIC, µg mL^{-1})**				
	0 x MIC	**0.5 x MIC**	**1 x MIC**	**2 x MIC**	**4 x MIC**
Phyllamyricin C (**1**)	99.0	89.7	87.1	83.7	81.5
Retrojusticidin B (**2**)	99.0	75.5	70.7	70.7	62.5
Phyllamyricin A (**3**)	99.0	62.5	62.5	62.5	0.0
Phyllamyricin E (**4**)	99.0	75.5	70.7	0.0	0.0
Justicidin B (**5**)	99.0	87.1	87.1	78.9	62.5
Phyllamyricin F (**6**)	99.0	70.7	70.7	62.5	0.0
Piscatorin (**7**)	99.0	78.9	62.5	0.0	0.0

A number of lignans have been evaluated as antifungal agents. For examples, three lignan compounds, alyterinate C, (+)-pinoresinol, and (+)-medioresinol, showed biological effects against a fungus-like organism *Pythium insidiosum* better than the antifungal drugs itraconazole and terbinafine [20]. Two aryltetralin lignans isolated from *Cordia exaltata* were found to be weak antifungal agents against several *Candida species* [21]. Ecbolin A (a furofuran type of lignan), a metabolite of *Exbolium viride*, showed moderate antifungal activity against twelve tested fungi [22]. (+)-Medioresinol has been known to posses antifungal properties against *C. albicans* via apoptosis mechanisms, namely oxidative stress and mitochondria dysfunction [23]. Besides, justicin B (**5**) from *P. piscotarum* [14], and phyllanthin, a dibenzylbutane lignan isolated from *P. niruri*, also exhibited antifungal properties against *Candida albicans* [24]. The antifungal properties of compound **5** reported in this work is consistent with the previous study [14] and our results therefore add more data on the beneficial effect of lignans from *Phyllanthus* species as antifungal agents. However, with reference to antifungal properties of lignans against *Fusarium* fungi, the data is rather very

limited. Pinoresinol (a furanoid lignan) and secoisolariciresinol (a dibenzilbutane lignan) have been shown to inhibit the growth and trichocetene biosynthesis in *F. graminearum* [25]. Lyoniside, a glucoside derivative of aryltetralin lignan isolated from *Vaccinium myrtillus* L. showed inhibition of mycelia growth of *F. oxysporum* [26]. However, eudesmin (a furanoid lignan) did not show antifungal activity against *F. oxysporum* [26]. Thus the effect of lignans on *Fusarium* fungi depends on the skeleton type of the lignan, and there is a possibility that arylnaphtalene lignans, such as compounds **1**-**7**, can act as potential anifungal agents against *F. oxysporum*.

The discovery of new conidia germination inhibitors would be valuable for the control of the diseases caused by pathogenic fungi. In addition, adequate treatment of mycotic infections is difficult since fungi are eukaryotic organisms with a structure and metabolism similar to that of the eukaryotic host [27, 28]. Furthermore, long-term treatment with commonly used antifungals such as amphotericin B has toxic effects and antifungal azoles are limited in their spectrum and efficacy, and their use may result in resistant strains [29-32].

CONCLUSION

Lignans isolated from *P. myrtifolius* might be potentially valuable as a natural and active compound against mycotic or fungal infections. *Phyllanthus* species has long been used in folk medicine in many countries as antimicrobials and/or antioxidants. The results of this study show the antifungal potential of seven lignans isolated from *P. myrtifolius*. Thus, the lignans isolated from the leaves of *P. myrtifolius* can be developed as natural antifungal agents. The toxicity of these compounds will be investigated in a future study.

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CONFLICT OF INTEREST STATEMENT

No conflicts of interest.

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