Antifungal Activity of Triterpenoids and Steroids Isolated from *Pinus merkusii* Bark Against *Phanerochaete chrysosporium*

Masendra, Brandon Aristo Verick Purba, and Ganis Lukmandaru

Abstract

The outer part of a tree, known as the bark, protects the internal tissues from external conditions and attacks from microorganisms. Its antifungal activities are due to the presence of chemicals in this bark. This study aimed to evaluating the toxicity of triterpenoids and steroids from the bark of *Pinus merkusii* against *Phanerochaete chrysosporium* (white-rot). The triterpenoids and steroids were isolated from *n*-hexane extract of the bark through column chromatography. Then, the antifungal activity was evaluated by measuring the growth rate of the fungus on potato dextrose agar (PDA) medium in a Petri dish. The isolation resulted in three triterpenoids (3β- methoxyserratt-14-en-21-one, serrate-14-en-3β,21β –diol, 3α,21β-dimethoxy-Δ14-serratene) and two steroids (β-sitosterol, Stigmast-4-en-3-one). There was more of antifungal activity for β-sitosterol and serrate-14-en-3β,21β –diol, due to presence of hydroxyl bond and their hydrophobicity.

Keywords: *Pinus merkusii*, bark, steroids, triterpenoids, antifungal, white-rot

Introduction

Bark is an important part, covering almost the whole part of a tree. According to Wittstock and Gershenzon (2002); Alfredsen *et al*. (2008); and Pásztory *et al*. (2016), it protects the inner part of the tree from extreme and other external conditions such fire, cold, herbivore, pathogens, as well as detrimental insects and fungi. One of the ways it defends the tree is by storing secondary metabolites toxic to its enemies. Previous researches showed that its phenolics and lipophilics components possess bioactivity actions againts various pests, disease-causing organisms, and wood decaying fungi such as white and brown-rot fungi (Wijayanto *et al*. 2015; Shreaz *et al*. 2016; Lomelí-Ramírez *et al*. 2016; Özgenç *et al*. 2017).

Pinus merkusii Jungh & de Vriese is a conifer of Pinaceae family, usually used as raw material in the production of pulp and paper, gum rosin as well as turpentine in Indonesia. Similarly, previous studies on *P. merkusii* bark showed the presence of phenolic and lipophilic extractives, also in its knots and stem wood (Wijayanto *et al*. 2015; Masendra *et al*. 2018a; Masendra *et al*. 2019; Masendra *et al*. 2020). In addition, Wijayanto *et al*. (2015) reported that the phenolic stilbenes of *P. merkusii* wood showed antifungal activity against *Trametes versicolor* (white-rot) and *Poria placenta* (brown-rot). Additionally, triterpenoids and steroids from *Astilbe myriantha* roots, *Ficus drupacea* bark and *Pallavicinia lyellii* have been reported to exhibit antifungal activity (Subhisha and Subramoniam 2005; Song *et al*. 2011; Yessoufoua *et al*. 2015). Furthermore, a previous work by Masendra *et al*. (2018b) showed the isolation of three triterpenoids and two steroids from the bark of *P. merkusii*. Based on this background, the aim of this study was to evaluate the toxicity of triterpenoids and steroids isolated from the bark of *P. merkusii* against *P. chrysosporium*. The use of the *P. chrysosporium* in this study due to the capability of this white-rot basidiomycete fungi to degrade lignin and also was used to test five species of woods in Brazil as their natural resistance (Oliveira *et al*. 2010).

Materials and Methods

Extraction and Isolation

Silica gel (60 N, spherical 63 μm to 210 μm; neutral Kanto Chemical Co., Inc., Tokyo, Japan) was applied for column chromatography with a glass column (40 × 2.5 cm inner diameter). Aluminum sheets pre-coated with silica gel 60 F254 (Merck, Kenilworth, NJ, USA) were used for thin-layer chromatography (TLC). The spots were visualized using ultraviolet (UV) light irradiation (λ = 254 and 360 nm) by spraying with vaniline-sulfuric acid, for color testing, followed by heating at 150 °C for 10 min.

The *P. merkusii* bark was subjected to extraction with *n*-hexane for two weeks under room temperature and then evaporated until air dry before weighing. The n-hexane extract with a yellow color was 1.59 g but after separation, 1.0 g was chromatographed into Silicon gel column chromatography (SiGCC). This resulted in 63.1 mg β-sitosterol, 13.9 mg Stigmast-4-en-3-one, 14.8 mg 3β-methoxyserratt-14-en-21-one, 71.0 mg serrate-14-en-3β,21β –diol, and 1.0 mg 3α,21β- dimethoxy-Δ14-serratene. The chemical structures of isolated compounds are shown in Figure 1.
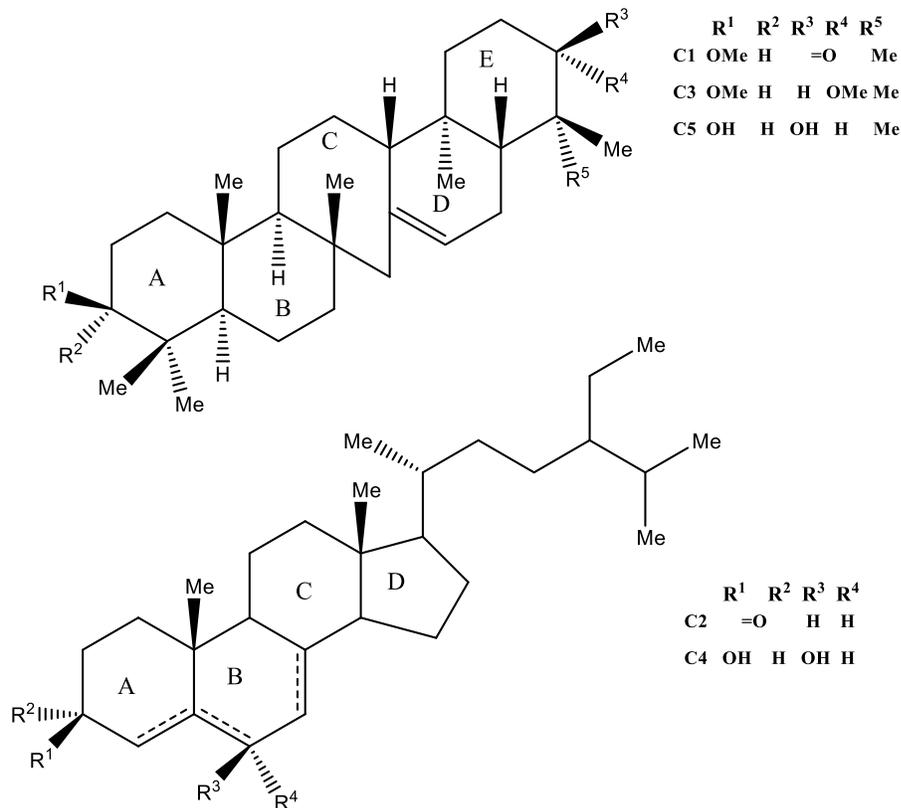
Figure 1. Chemical structure of isolated compounds from *P. merkusii* bark; **C1**: 3β- methoxyserratt-14-en-21-one, **C2**: stigmast-4-en-3-one, **C3**: 3α,21β- dimethoxy-Δ14-serratene, **C4**: β-sitosterol, and **C5**: Serrate-14-en-3β,21β–diol.

3β- Methoxyserratt-14-en-21-one (**C1**). EI-MS m/z 454 (M+; $C_{31}H_{50}O_2$, 43), 221 (100), 218 (73), and 135 (63) The ^{1}H-NMR (CDCl₃): δ 0.93 (3H, s, H-23-Me), 0.73 (3H, s, H-24-Me), 0.77 (3H, s, H-25-Me), 0.80 (3H, s, H-26-Me), 0.90 (3H, s, H-28-Me), 1.02 (3H, s, H-29-Me), 1.06 (3H, s, H-30-Me), 2.60 (1H, dd, *J* = 11.7 and 4.1 Hz, H-3α), 2.73 (1H, dt, *J* = 14.8 and 5.5 Hz, H-20), 3.33 (3H, s, H-3-OMe), and 5.35 (1H, brs, H-15). ^{13}C-NMR (CDCl₃): δ 38.5 (C-1), 22.3 (C-2), 88.4 (C-3), 38.2 (C-4), 56.2 (C-5), 18.7 (C-6), 45.1 (C-7), 38.9 (C-8), 56.2 (C-9), 36.1 (C-10), 25.5 (C-11), 27.2 (C-12), 62.7 (C-13), 138.3 (C-14), 122 (C-15), 24.4 (C-16), 51.2 (C-17), 37.1 (C-18), 38.3 (C-19), 34.8 (C-20), 217.1 (C-21), 47.6 (C-22), 15.7 (C-23), 19.8 (C-24), 28.1 (C-25), 16.2 (C-26), 55.9 (C-27), 12.9 (C-28), 24.5 (C-29), 21.6 (C-30) 57.5 (OMe).

Stigmast-4-en-3-one (**C2**). The EI-MS m/z 412 (M+; $C_{29}H_{48}O$, 39), 397 (8), 370 (14), 289 (22), 229 (38), and 124 (100). The ^{1}H-NMR (CDCl₃) results were as follows: δ 1.49, 1.24 (each 1 H, m, H-1), 2.36, 2.25 (each 1 H, m, H-2), 5.70 (1H, br, s, H-4), 2.02, 1.90 (each 1H, m, H-6), 1.18, 1.42 (each 1 H, m, H-7), 1.41 (1H, m, H-8, H-9), 1.56, 1.27 (each 1H, m, H-11), 1.57, 1.31 (each 1 H, m, H-12), 1.02 (1H, m, H-14), 1.62, 1.35 (each 1 H, m, H-15, H-16), 1.13 (1H, m, H-17), 0.69 (3H, s, H-18), 1.16 (3H, s, H-19), 1.65 (1H, s, H-20), 0.84 (3H, d *J* = 6.9 Hz, H-21), 1.27, 1.27 (each 1 H, m,

H-22, H-23), 1.48 (1H, m, H-24), 1.83 (1H, m, H-25), 0.80 (3H, d, *J* = 6.9 Hz, H-26), 0.78 (3H, d, *J* = 6.9 Hz, H-27), 1.56, 1.56 (each 1 H, m, H-28), 0.9 (3H, t, *J* = 6.9 Hz, H-29). ^{13}C-NMR (CDCl₃): δ 35.7 (C-1), 33.9 (C-2), 199.8 (C-3), 123.8 (C-4), 171.9 (C-5), 32 (C-6), 29.7 (C-7), 35.6 (C-8), 53.8 (C-9), 38.6 (C-10), 21 (C-11), 39.6 (C-12), 42.4 (C-13), 55.8 (C-14), 24.2 (C-15), 32.9 (C-16), 56 (C-17), 12 (C-18), 17.4 (C-19), 36.1 (C-20), 18.7 (C-21), 34 (C-22), 26 (C-23), 45.8 (C-24), 28.2 (C-25), 19.8 (C-26), 19 (C-27), 23 (C-28), 11.9 (C-29).

3α,21β- Dimethoxy-Δ14-serratene (**C3**). (EI-MS) m/z 470 (M+; $C_{32}H_{54}O_2$, 54), 455 (35), 438 (18), 423 (18), 234, (52), 221, (81), 189 (100), 149 (20), 135 (72), and 147 (43). The ^{1}H-NMR (CDCl₃) results were as follows: δ 0.93 (3H, s, H-23-Me), 0.65 (3H, s, H-24-Me), 0.72 (3H, s, H-25-Me), 0.78 (3H, s, H-26-Me), 0.80 (3H, s, H-28-Me), 0.92 (3H, s, H-29-Me), 0.93 (3H, s, H-30-Me), 3.33 (3H, s, 21-OMe), 2.03 (3H, s, H-3-OMe), 2.62 (1H, dd, *J* = 11.9 and 4.1 Hz, H-3-α), 5.28 (1H, brs, H-15).

β-Sitosterol (**C4**). The EI-MS m/z 414 (M+; $C_{29}H_{50}O$, 91), 396 (43), 81 (85), 55 (100). The ^{1}H-NMR (CDCl₃) results were as follows: δ 1.13, 1.13 (2H, m, H-1), 1.58, 1.23 (2H, m, H-2), 3.5 (1H, m, H-3), 2.23, 1.97 (2H, m, H-4), 5.33 (1H, m, H-6), 2.15, 1.97 (2H, m, H-7), 1.48 (1H, m, H-8), 1.44 (1H, m, H-9), 1.51, 1.22 (2H, m, H-11), 1.56, 1.47 (2H, m, H-12),

1.41 (1H, m, H-14), 1.63, 1.46 (2H, m, H-15, H-16), 1.47 (1H, m, H-17), 1.04 (3H, m, H-18), 1.05 (3H, m, H-19), 1.64 (1H, m, H-20), 0.98 (3H, d, J = 6.9 Hz, H-21), 1.25 (2H, m, H-22, H-23), 1.46 (1H, m, H-24), 1.81 (1H, m, H-25), 0.81 (3H, d, J = 4.8 Hz, H-26), 0.89 (3H, d, J = 4.8 Hz, H-27), 1.49 (2H, m, H-28), 0.90 (3H, m, H-29). ^{13}C-NMR (CDCl$_3$): δ 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.2 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9 (C-7), 31.8 (C-8), 50.1 (C-9), 36.5 (C-10), 22.6 (C-11), 39.7 (C-12), 42.3 (C-13), 56.7 (C-14), 24.3 (C-15), 26 (C-16), 56 (C-17), 11.8 (C-18), 19 (C-19), 36.1 (C-20), 18.7 (C-21), 33.9 (C-22), 26 (C-23), 45.8 (C-24), 29.1 (C-25), 19.8 (C-26), 21 (C-27), 23 (C-28), 12 (C-29).

Serrate-14-en-3β,21β–diol (**C5**). EI-MS m/z 442 (M⁺; C$_{30}$H$_{50}$O$_2$, 33), 427 (29), 409 (16), 391, 220 (26), and 207 (100). The ^{1}H-NMR (CDCl$_3$) results were as follows: δ 0.94 (3H, s, H-23-Me), 0.74 (3H, s, H-24-Me), 0.77 (3H, s, H-25-Me), 0.81 (3H, s, H-26-Me), 0.91 (3H, s, H-28-Me), 1.02 (3H, s, H-29-Me), 1.06 (3H, s, H-30-Me), 3.17 (1H, dd, J = 11.7 and 4.1 Hz, H-3-$β$), 3.43 (1H, brs, H-21-$β$), and 5.30 (1H, brs, H-15). ^{13}C-NMR (CDCl$_3$): δ 38.5 (C-1), 25.3 (C-2), 78.9 (C-3), 38.1 (C-4), 55.7 (C-5), 18.9 (C-6), 45.1 (C-7), 38.9 (C-8), 56.8 (C-9), 35.9 (C-10), 25.4 (C-11), 27.1 (C-12), 62.8 (C-13), 138.5 (C-14), 122 (C-15), 24 (C-16), 43.3 (C-17), 37.1 (C-18), 31.2 (C-19), 27.5 (C-20), 76.2 (C-21), 37.4 (C-22), 15.7 (C-23), 19.8 (C-24), 28.1 (C-25), 15.4 (C-26), 56.2 (C-27), 13.3 (C-28), 27.7 (C-29), 21.8 (C-30).

GC-MS Analysis

Gas chromatography-mass spectrometry (GC-MS) data were collected with the instrument procured from Shimadzu, Kyoto, Japan, with the following conditions: column temperature from 50 °C (1 min) to 320 °C at 5 °C/min; injection temperature of 250 °C; detection temperature of 320 °C; acquisition mass range of 50 to 800 amu using helium as the carrier gas. The specification of column: DB-1 capillary column (30 × 0.25 mm inner diameter and 0.25 μm; GL Sciences, Tokyo, Japan). The ^{13}C and ^{1}H nuclear magnetic resonance (NMR) were determined by a JEOL ECZ-400 spectrometer (JEOL, Tokyo, Japan). Then, the NMR spectra were recorded using standard JEOL pulse sequences at 400 MHz and 100 MHz for ^{1}H and ^{13}C, respectively.

Antifungal Activity

The toxicity of the isolated compounds against the growth of *P. chrysosporium* were investigated through literature of Lukmandaru (2013). Exactly 1 mg/ml concentration of each sample was prepared and a solution of 300 μl was placed on the surface of 20 ml PDA medium with 12.3 mg/ cm^2 concentration in a 9 cm diameter petri dish. The extract solution was allowed to stand for 1 h air dry before inoculation. The blank was performed to the sample solvent without extract and each sample was conducted in three replications. The lapachol analytical standard (Aldrich, Germany) was used in this assay as a positive control. Then, the growth rate of sample was calculated using equation 1:

$$\text{Growth rate (\%)} = A_1 / A_0 \times 100\% \qquad (1)$$

Where $A = \pi \times (d/2)^2$, d = diameter of sample growth, A_0 is growth rate of blank and A_1 is growth rate of sample.

Statistical Analyses

One-way analysis of variance (ANOVA) were performed on the results of the growth rate using SPSS version 20 (IBM, New York, USA) with 95% confidence level. Significant results were further subjected to post-test Tukey HSD.

Results and Discussion

Extractive Content and Constituents of *n*-hexane Extract

The extraction from the *P. merkusii* bark by *n*-hexane produced 1.59 g of extractive content on the basis of dry bark sample. In comparison, the extractive content of *P. merkusii* bark (0.16%) was lower than previous works i.e *P. echinata* bark of 2.6%, P. *taeda* of 1.7% (Fengel and Wegener 1989), and *P. pinea* of 2.1% (Nunes *et al*. 1999). According to Masendra *et al*. (2018a), the constituent of *n*-hexane extracts contained serratene triterpenes and sterols. The chromatogram of *n*-hexane extract of *P. merkusii* in Figure 2, showed that β-sitosterol (**C4**) was the major compound followed by 3β-methoxyserratt-14-en-21-one (**C1**). However, stigmast-4-en-3-one (**C2**), 3α,21β-dimethoxy-Δ14-serratene (**C3**) and serrate-14-en-3β,21β-diol (**C5**) were present in low concentration.
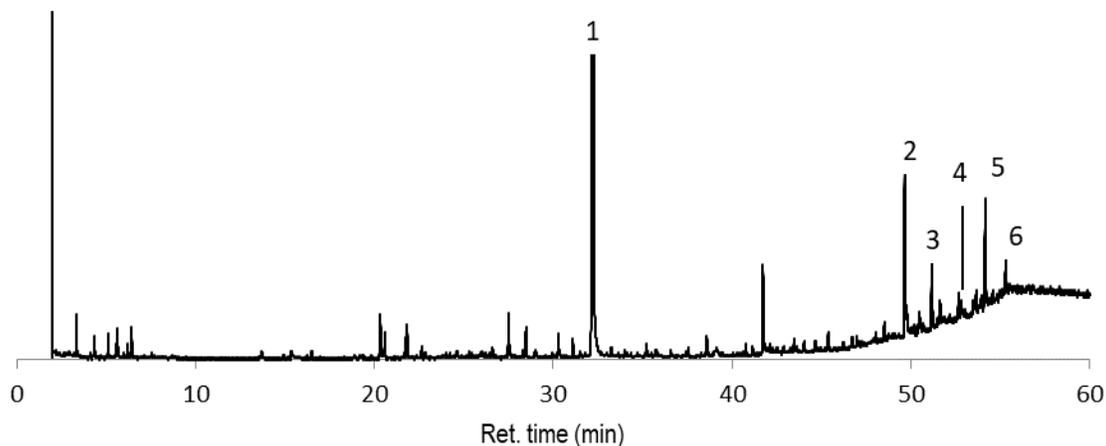
Figure 2. Chromatogram of *n*-hexane extract of *P. merkusii* bark; 1. Internal standard (heneicosane (ret. time: 32.26 min), 2. β-sitosterol (49. 65 min), 3. Stigmast-4-en-3-one (51.17), 4. 3α,21β-dimethoxy-Δ14-serratene (53.70), 5. 3β methoxyserratt-14-en-21-one (54.16), 6. Serrate-14-en-3β,21β-diol (55.29).

Antifungal Activity of Isolated Compounds

The antifungal activity of isolated compounds was measured with reference to the growth rate of *P. chrysosporium*. The fungus growth on PDA medium as represented in Figure 3, showed a cycle shape and fungus spread from the center of petri dish to the surface of PDA. Also, the results of the one-way ANOVA showed significant difference (p=<0.01) among the compound. The growth rates of **C1**-**C3,** as shown in Figure 4, were 100% with no antifungal activity. However, there were significantly higher growth inhibition rates in β-sitosterol (C4) and serrate-14-en-3β,21β-diol (C5) with inhibition of 45.5% and 60.2%, respectively, but still low compared with lapachol standard with 26.7%. Also, the positive control with lapachol was two times higher than β-sitosterol and serrate-14-en-3β,21β-diol.
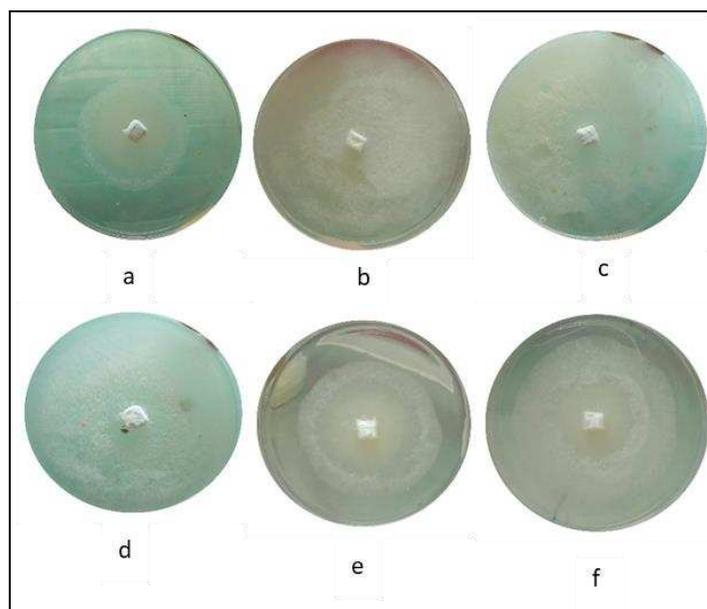

Figure 3. Growth rate of fungus performance of standard of lapachol (a), **C1** (b), **C2**, (c), **C3** (d), **C4** (e), **C5** (f)
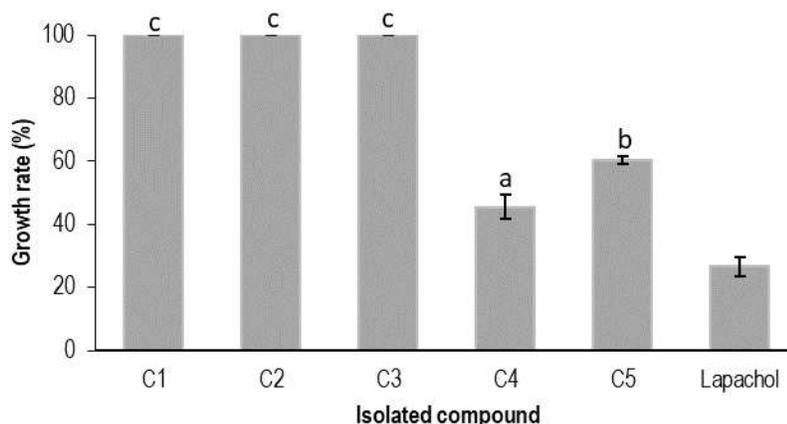
Figure 4. Antifungal activity of isolated compounds of *P. merkusii* bark. Different letter (a, b, etc.) indicates significant difference at 0.05 level by Tukey HSD test

In this research, no literature was found on the antifungal activity of triterpenoids and steroids from *P. merkusii* bark against *P. chrysosporium*. However, the antifungal activity of other compounds have been reported from other species such as 3b,6b,24-trihydroxyurs-12-en-27-oic acid from *Astilbe myriantha* showing strong antifungal activity against *Colletotrichum gloeosporioides* (Song *et al*. 2011), epilupeol acetate from *Ficus drupace* against *Aspegillus versicolor* (Yessoufoua *et al*. 2015), and β-sitosterol from *Senecio lyratus* against *Fusarium spp.* (Kiprono *et al*. 2000).

Correlation between Antifungal Activity and Chemical Structure of Isolated Compounds

It was observed that β-sitosterol and serrate-14-en-3β,21β-diol affected the growth rate of *P. chrysosporium*. Also, among the isolated samples, only β-sitosterol and serrate-14-en-3β,21β-diol have hydroxyl bond (-OH) in their structures as shown in Figure 1. This is an indication that the hydroxyl bond, which determine the lipophilicity and hydrophobicity of the compounds, affect the antifungal activity.

The hydroxyl bond was located in A and B rings in β-sitosterol, but in A and E rings in serrate-14-en-3β,21β-diol. Hence, with β-sitosterol, the fungal growth rate was lower compared with serrate-14-en-3β,21β-diol. This means that the hydroxyl position and hydrophobicity of A and B ring were stronger in inhibiting the growth of *P. chrysosporium* compared with the bond in A and E rings. In line with these findings, Sekine *et al*. (2009), and Zengin and Baysal (2014) also reported that the hydrophobicity and hydroxyl position of terpenes, latifolin, and the derivative products affected the antibacterial and antifungal activity by inhibiting the growth of brown- and white-rot. Additionally, a recent study by Konuk and Ergüden (2020) reported that the –OH position and hydrophobicity of phenolic terpenoids affected the

disruption of cell membrane integrity of *Saccharomyces cerevisiae*.

Ecological Role and the Potential Use of Triterpenoids and Steroids

The terpenoid compounds in *P. merkusii* bark was reported to play a vital role in preventing living tissues from chemical deterioration (Seki *et al*. 2012). In addition, the presence of triterpenoids and steroids in this bark, especially those with hydroxyl bond in their ring are more effective against *P. chrysosporium* (white-rot). Therefore, high concentration of triterpenoids and steroids in the bark of a tree protect its living tissues from damage due to fungal attacks.

Due to the impact of microorganisms such as bacterial and white-rot fungus attacks, there is need for further research on natural wood preservatives. The use of natural preservatives could reduce the negative effects of chemicals in the air. According to Tascioglu *et al*. (2013); Smith *et al*. (1989); and Lin *et al*. (2007), some plants extracts could be processed into alternative wood preservatives due to their actions against microorganisms' attacks. Hence, the accumulation of triterpenoids and steroids in the bark of *P. merkusii*, could be used as alternative wood preservatives.

Conclusion

Three triterpenoids (3β- methoxyserratt-14-en-21-one, serrate-14-en-3β,21β –diol, and 3α,21β- dimethoxy-Δ14-serratene) and two steroids (β-sitosterol and stigmast-4-en-3-one) were isolated and identified from the bark of *P. merkusii*. Among all these isolated samples, β-sitosterol and serrate-14-en-3β,21β –diol showed antifungal activity with growth inhibition rates of 45.5% and 60.2%, respectively. However, the hydrophobicity and presence of hydroxyl bond in β-sitosterol and serrate-14-en-3β,21β –diol affected the antifungal activity. Hence, there is need for further research

on the correlation between bioactivity and chemical structure of these two compounds with other fungi groups.

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Masendra, Brandon Aristo Verick Purba, Ganis Lukmandaru
Department of Forest Products Technology, Faculty of Forestry, Universitas Gadjah Mada,
Jl. Agro No.1, Bulaksumur, Yogyakarta 55281, Indonesia
Tel.   : +6274 550541
Fax.   : +6274 550541
Email   : glukmandaru@ugm.ac.id