



An Evaluation of the Antifungal and Antioxidant Activity of *Pinus merkusii* Bark Ethyl Acetate Extract

(Evaluasi Aktivitas Antijamur dan Antioksidan dari Ekstrak Etil Asetat Kulit Kayu *Pinus merkusii*)

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ABSTRACT

Pinus merkusii is the only species in the Pinaceae family to grow in Southeast Asia, including Indonesia, and serves as a source of wood and oleoresins. During the utilization of this pine, the bark is discarded as residue; however, this residue is possibly a potential source of antifungal and antioxidant agents, due to the polyphenol contents. This study, therefore, aims to investigate the antifungal and antioxidant compounds present in the bark of *P. merkusii*. The ethyl acetate extracts of *Pinus merkusii* were fractionated to obtain five fractions (Fr. A- Fr. E) and the bioactivity of each fraction was observed. Subsequently, the growth inhibition against *Phanerochaete chrysosporium*, was evaluated for antifungal activity, while DPPH (1,1-diphenyl-2-picrylhydrazyl) method was used to ascertain antioxidant activity. According to the GC-MS analysis, the ethyl acetate fractions contain triterpenoids and steroids as the dominant compounds as well as minor amounts of mono-sesquiterpenoids and hydrocarbons. The highest growth inhibition exhibited by Fr. C- Fr. E (>60%) indicates triterpenoids and steroids act as a fungal inhibitors. Meanwhile, the five fractions exhibited low antioxidant activity because the steroids and triterpenoids present are possibly ineffective DPPH inhibitors. Also, the antifungal and antioxidant activity R₂ values were discovered to correlate 0.3, indicating a low positive correlation.

INTISARI

Pinus merkusii merupakan spesies dari keluarga Pinaceae yang tumbuh di Asia Tenggara termasuk Indonesia. *Pinus merkusii* dimanfaatkan untuk menghasilkan kayu dan getah, namun dalam pemanfaatannya masih meninggalkan residu berupa kulit kayu pinus yang diperkirakan berpotensi sebagai anti-jamur dan antioksidan disebabkan kandungan fenolatnya. Penelitian ini bertujuan untuk menginvestigasikan kandungan kimia kulit kayu *P. merkusii* serta antijamur dan antioksidannya. Metode penelitian dilakukan dengan ekstraksi kulit kayu dengan etil asetat dan ekstrak etil asetat difraksinasi menggunakan kromatografi kolom sehingga dihasilkan lima fraksi (Fr. A-E). Uji antijamur dilakukan dengan menggunakan spesies *Phanerochaete chrysosporium*, sementara uji antioksidan menggunakan metode DPPH (1,1-diphenyl-2-picrylhydrazyl). Hasil identifikasi senyawa kimia dengan

GC-MS kelima fraksi etil asetat mengandung komponen yang didominasi oleh steroid dan triterpenoid, sedangkan komponen monoterpena, sesquiterpena, dan hidrokarbon dideteksi dalam jumlah kecil. Pengujian anti-jamur menunjukkan Fr. C- Fr. E dengan hasil inhibisi tinggi (>60%) diduga karena adanya komponen dominan berupa steroid dan triterpenoid. Hasil uji antioksidan menunjukkan kelima fraksi memiliki nilai inhibisi yang rendah diduga komponen steroid dan triterpenoid tidak berperan baik sebagai penghambat DPPH. Uji lebih lanjut yaitu korelasi antara nilai inhibisi antijamur dan antioksidan menghasilkan korelasi yang rendah ($R^2 = 0.3$).

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Introduction

Pinus merkusii is one of the species belonging to Pinaceae family and native to Southeast Asia, including Indonesia. This pine has been planted in Perhutani enterprise area for oleoresins production and the lumber is used for the construction of a building and other long fiber materials. Perhutani planted *P. merkusii* across an area of 476,126 ha in Java Island. Thus, while utilizing this pine in Indonesia, a large quantity of the pine bark is discarded as residue. However, the high lipophilic and polyphenolic contents of the pine bark (Ku et al. 2007), increase the bark's potential for phytomedicine would be increased. In addition, plants with high lipophilic and hydrophilic content are also suggested to be a source of natural wood preservatives (Tascioglu et al. 2013; Smith et al. 1989; Lin et al. 2007). Living tissues in the tree are protected by the bark from damage due to extreme external conditions, including low and high temperature, microorganism as well as herbivore attacks, and this ability is well correlated with the presence of phenolic and lipophilic compounds in the bark (Wittstock and Gershenson 2002; Alfredsen et al. 2008; Seki et al. 2012).

Previous studies showed the wood and bark of *P. merkusii* contain phenolic and lipophilic substances (Wijayanto et al. 2015; Masendra et al. 2018a; Masendra et al. 2018b; Masendra et al. 2019; Masendra et al. 2020). Furthermore, Wijayanto et al. (2015) reported the application of *P. merkusii* wood extracts'

antioxidant and antifungal activities against white -rot and brown -rot fungi. However, the information on the antifungal and antioxidant activity exhibited by the bark of *P. merkusii* is still limited. In addition, the *n*-hexane and methanol extract of *P. merkusii* bark bioactivity were previously conducted (Masendra et al. 2020a; Masendra et al. 2020b). Therefore, this study aimed to investigate the constituents of *P. merkusii* bark extract and the respective bioactivity against *P. chrysosporium* and DPPH.

Materials and Method

Sample Collection, Extraction, and Fractionation

The bark sample (1 kg) was collected from Magelang, Central Java, Indonesia, grounded into a powder then extracted with *n*-hexane, ethyl acetate (EtOAc), and methanol, successively. Each extraction was performed for 2 weeks at room temperature. Subsequently, the solvent was evaporated and the dry extracts were calculated as a percentage of the dry bark sample. The EtOAc extract was subjected to column chromatography separation using eluent *n*-hexane and change polarity with EtOAc. Meanwhile, 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 cm \times 2.5-cm inner diameter), and aluminum sheets coated with silica gel 60 F254 (Merck, Kenilworth, NJ, USA) were used for thin-layer chromatography (TLC). The spots were visualized under ultraviolet (UV)

irradiation ($\lambda = 254$ nm and 360 nm) by spraying with vanillin-sulfuric acid (for colour testing), followed by heating at 150 °C for 10 min. In this study, five fractions, Fraction A (Fr. A) to Fr. E, were obtained.

Antifungal Activity

The *Phanerochaete chrysosporium* (white-rot) was used to determine antifungal activity. Growth inhibition of the obtained fractions of *P. merkusii* bark EtOAc extract against *P. chrysosporium* was evaluated according to Lukmandaru (2013). For this purpose, 300 μ l (1000 ppm) of the Fr. A – Fr. E samples were spread on the surface of 20 ml potato dextrose agar (PDA; Aldrich, Germany) medium in a Petri dish with a diameter of 9 cm of diameter, with the final concentration of 12.3 mg/ cm². Subsequently, all solvents were dried at room temperature for 1 hour then inoculated with *P. chrysosporium*. The sample without extract was used as blank, while a standard of lapachol (Aldrich, Germany) was used as the positive control. Each experiment was conducted in three replications, and the sample's growth inhibition was calculated using the equation below.

$$\text{Growth inhibition} = 100 \times ((A_0 - A_1) / A_0) \quad (1)$$

Where, $A = \pi \times (d/2)^2$, d = diameter of sample growth, A_0 denotes the growth inhibition of blank and A_1 signifies the growth inhibition of sample.

DPPH/ Antioxidant Activity

The antioxidant activity assay was performed using the method described by Baba and Malik (2014). For this assay, the sample (Fr. A- Fr. E) of 0.1 ml extract in methanol with a concentration of 1 mg/ml, was mixed and reacted with 3 ml of 0.1 mM DPPH (Aldrich, Germany). The reaction was incubated at room temperature for 30 minutes. Meanwhile, the standard of quercetin (Aldrich, Germany) was used as the positive control. Subsequently, the sample

solution's absorbance at 517 nm was recorded and the antioxidant activity was calculated using the equation below.

$$\text{DPPH scavenged (\%)} = 100 \times ((A_0 - A_1) / A_0) \quad (2)$$

Where, A_0 and A_1 denote the absorbance of the blank and the sample, respectively.

GC-MS Analysis

For this analysis, 1 μ l of Fr. A – Fr. E samples (1000 ppm) was prepared for the direct injection method. The gas chromatography-mass spectrometry (GC-MS) data were collected with a GCMS-QP2010 (Shimadzu, Kyoto, Japan) with conditions of 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 amu to 800 amu, using helium as the carrier gas. Meanwhile, the column specifications were DB-1 capillary column (30 mm \times 0.25-mm inner diameter and 0.25 μ m; GL Sciences, Tokyo, Japan). Subsequently, the mass spectra of the sample were then compared to NIST library and the literature (Masendra et al. 2018b).

Statistical Analysis

One-way analysis of variance (ANOVA) was conducted on the growth rate test results, using SPSS statistics 20 (IBM, New York, USA) with 95% confidence level. The significant results were further analysed using post-test Tukey HSD.

Results and Discussion

Extraction Yield

This paper does not report the *n*-hexane and methanol extract contents; however, the EtOAc extraction resulted in 3.159 g or less than 0.5% of the initial sample. The EtOAc extract (1.03 g) was fractionated through column chromatography and

five fractions (Fr. A- Fr. E) were obtained. Subsequently, the Fr. A- Fr. D were collected in a concentration range of 7-10%, while Fr. E was collected in a high concentration of 50.1% (Figure 1). A total of about 87.3% or 0.89 g of EtOAc extract was recovered. The extraction yields did not increase with an increasing quantity of EtOAc solvent in Fr. B- Fr. D, however, this was not the case with Fr. D - Fr. E, as the extract of Fr. E was five times higher compared to Fr. D, due to higher compounds dissolving in EtOAc solvent. This pattern of extraction yield was different compared to the isolation of EtOAc extract from teak wood conducted by Lukmandaru and Ogiyama (2005), where the teak wood extract dissolved more in a 1:1 volume mixture of benzene and EtOAc.

Identification and Characterization of Fr. A- Fr. E

According to the GC-MS analysis of the five EtOAc extract fractions, Fr. A contained compounds 2, 4, and 7, Fr. B contained compounds 5 and 8, Fr. C contained compounds 6, 7, and 8, Fr. D contained compounds 5 and 7, while Fr. E contained compounds 6, 7, and 9 (Figure 2).

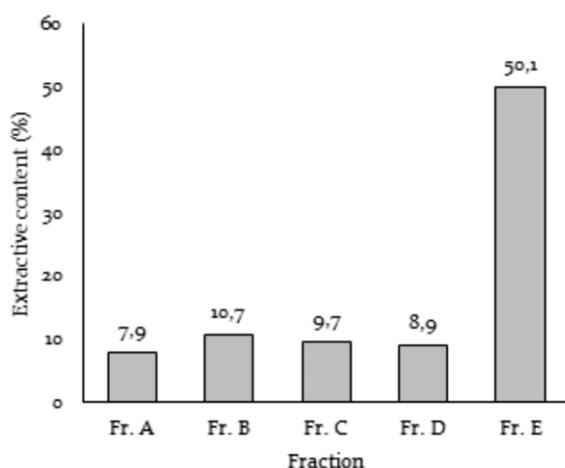


Figure 1. Yield of fractionation of EtOAc extract of *P. merkusii* bark; eluting solvent of Fr. A (*n*-hexane- EtOAc, 10/0, v/v), Fr. B (*n*-hexane- EtOAc, 8/2, v/v), Fr. C (*n*-hexane- EtOAc, 7/3, v/v), Fr. D (*n*-hexane- EtOAc, 5/5, v/v), Fr. E (*n*-hexane- EtOAc, 0/10, v/v).

Gambar 1. Hasil rendemen fraksinasi dari ekstrak etil asetat kulit kayu *P. merkusii*; eluen Fr. A (*n*-heksana- EtOAc, 10/0, v/v), Fr. B (*n*- heksana - EtOAc, 8/2, v/v), Fr. C (*n*- heksana - EtOAc, 7/3, v/v), Fr. D (*n*- heksana - EtOAc, 5/5, v/v), Fr. E (*n*- heksana - EtOAc, 0/10, v/v).

The compounds 1, 2, 3, 4, and 6 were compared with NIST library and a similarity index above 80% was obtained. Compounds 1 and 2 were considered as mono and sesquiterpene (α - terpeneol and caryophyllenyl alcohol), 3 and 4 were suggested to be fatty acid and hydrocarbon (docosanoic acid and heneicosane), while compound 6 was implied to be a steroid (25-hydroxycholesterol).

Meanwhile compounds 5, 7, 8, and 9 were compared with literature (Masendra et al. 2018b). Compound 5 had an MS peak at *m/z* 414 with other fragmentation of *m/z* 397, 371, 355, 289, 229, 124 (base peak), 107, and 55. These fragmentations were similar to stigmas-4-en-3-one steroid. In addition, compounds 7, 8, and 9 showed MS peaks at *m/z* 470, 454, and 442, and compound 7 showed other fragmentation of *m/z* 455, 438, 234, and a base peak at *m/z* 189. The results of compound 7 were similar to 3 α ,21 β -dimethoxy-d14-serratene. Meanwhile, compound 8 showed other fragmentation of *m/z* 221 (base peak), 218, 135, 59, and 55, and these fragment patterns are similar to 3 β -methoxyserratt-14-en-21-one. The last compound (9) was concluded to be serrate-14-en-3 β ,21 β -diol, due to fragmentation at *m/z* 427, 391, 220, and 207 (base peak).

Similarly, the presence of compounds 1 and 2 as mono and sesquiterpene were also detected in the bark of *P. silvestris*, *P. elliotii*, and *P. montezumae* (Norriin and Winnel, 1972; Masendra et al. 2018a), while compounds 3 and 4 as fatty acids and hydrocarbon were reported to be present in the bark of *P. elliotii*, *P. insularis*, *P. montezumae*, *P. oocarpa*, and *P. caribeeae* (Masendra et al. 2018a). Furthermore, compounds 5- 9 as steroids and triterpenoids was observed in *P. armandii* (Fang et al. 1991), *P. luchuensis* (Yamamoto et al. 2011), *P. caribeeae*, *P. oocarpa*, *P. elliotii*, *P. monemumae*, and *P. insularis* (Masendra et al. 2018a).

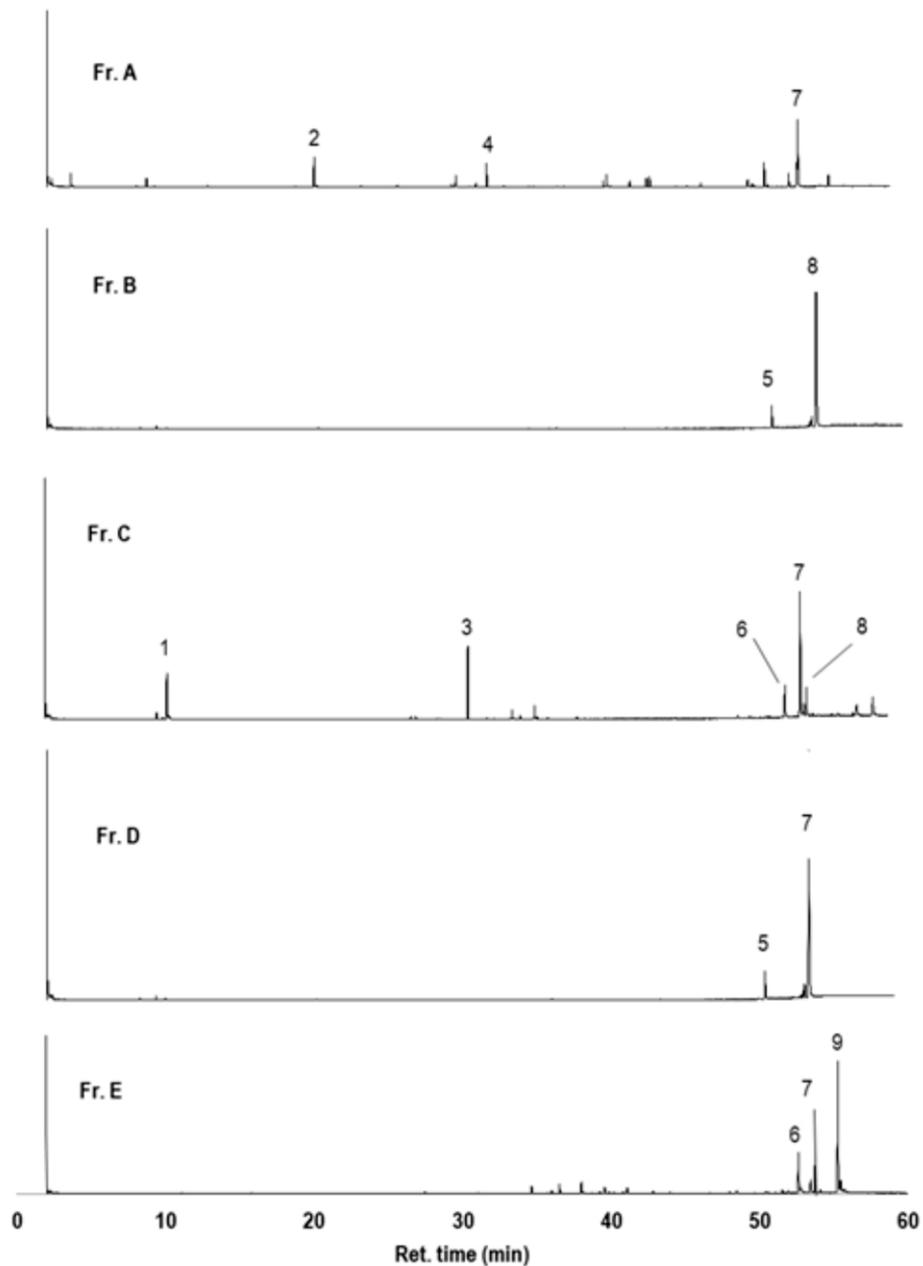


Figure 2. GC-MS chromatogram of Fr. A- Fr. E; compound 1. α -terpineol (ret. Time: 10.4 min), 2. caryophyllenyl alcohol (20.3 min), 3. docosanoic acid (31.1 min), 4. heneicosane (32.2 min), 5. stigmast-4-en-3-one (51.1 min), 6. 25-hydroxycholesterol (52.9 min), 7. $3\alpha,21\beta$ -dimethoxy-d₁₄-serratene (53.7 min), 8. 3β -methoxyserrat-14-en-21-one (54.3 min), 9. serrate-14-en- $3\beta,21\beta$ -diol (55.3 min)

Gambar 2. Kromatogram GC-MS dari Fr. A- Fr. E; senyawa 1. α -terpineol (waktu retensi: 10,4 menit), 2. caryophyllenyl alcohol (20,3 menit), 3. Asam dokosanoat (31,1 menit), 4. heneikosana (32,2 menit), 5. stigmast-4-en-3-one (51,1 menit), 6. 25-hydroxycholesterol (52,9 menit), 7. $3\alpha,21\beta$ -dimethoxy-d₁₄-serratene (53,7 menit), 8. 3β -methoxyserrat-14-en-21-one (54,3 menit), 9. serrate-14-en- $3\beta,21\beta$ -diol (55,3 menit)

Antifungal Activity

Figures 3 and 4 show the growth inhibition exhibited by each fraction against *P. chrysosporium* white-rot fungi. Fraction A showed the lowest growth inhibition ($26.8 \pm 11.5\%$), while the strongest growth inhibition was exhibited by Fraction E ($66.8 \pm 6.8\%$),

and this is slightly below the positive control, lapachol ($73.3 \pm 3.0\%$). In addition, one-way ANOVA of the fractions resulted in a significance difference ($p < 0.01$), while Tukey HSD test showed Fr. C- Fr. E exhibited significantly higher growth inhibition, compared to Fraction A.

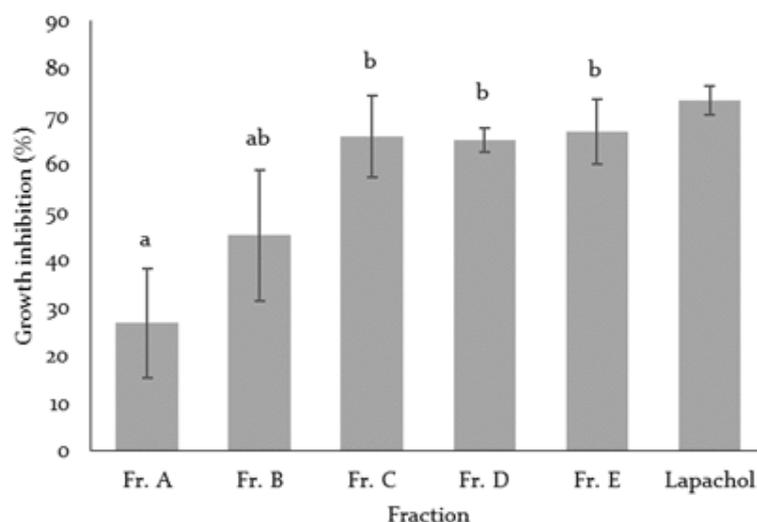


Figure 3. Antifungal activity of EtOAc fractions of *P. merkusii* bark. Different letters on the histogram (a, b, etc.) indicate a significant difference of growth inhibition by Tukey HSD at 0.05 level

Gambar 3. Aktivitas antijamur dari fraksi-fraksi EtOAc kulit kayu *P. merkusii*. Huruf yang berbeda pada histogram (a, b, dan seterusnya) menunjukkan perbedaan nyata nilai penghambatan pertumbuhan jamur pada uji lanjut Tukey HSD taraf nyata 0,05

An antifungal study of latifolin derivatives against white-rot (*Trametes versicolor*) showed antifungal inhibition was attributed to the hydrophobicity of hydroxyl and methoxy groups (Sekine et al. 2009). Furthermore, the presence of steroid (25-hydroxycholesterol and stigmast-4-en-3-one) as well as triterpenoid (3 α ,21 β -dimethoxy-d14-serratene, 3 β -methoxyserratt-14-en-21-one and serrate-14-en-3 β ,21 β -diol) structures in Fr. C- Fr. E possibly correlate to inhibition of *P. chrysosporium*. In Fr. C- Fr. E, the methoxy group occurred in the structures of 3 β -methoxyserratt-14-en-21-one and 3 α ,21 β -dimethoxy-d14-serratene, while the hydroxyl group occurred in serrate-14-en-3 β ,21 β -diol and 25-hydroxycholesterol. However, the presence of 3 α ,21 β -dimethoxy-d14-serratene and 3 β -methoxyserratt-14-en-21-one in Fr. A and Fr. B did not support fungal growth inhibition, thus, suggesting only the hydroxyl groups in Fr. C- Fr. E steroid and triterpenoid supported the inhibition of white-rot (*P. chrysosporium*) growth.

In addition, the antifungal properties of triterpenoids and steroids from other compounds were also reported. The triterpene of 3b,6b,24-trihydroxyurs-12-en-27-oic acid exhibited strong

antifungal properties against *Colletotrichum gloeosporioides* (Song et al. 2011), the triterpene of epilupeol acetate also exhibited antifungal properties against *Aspegillusversicolor* (Yessoufou et al. 2015), and the steroid of β -sitosterol showed antifungal properties against *Fusarium spp.* (Kiprono et al. 2000). The antifungal evaluation in this study (Fr. C- Fr. E) was indicated to be affected by several compounds. Therefore, compounds 5- 9 need to be purified to clarify the compound acting as antifungal towards *P. chrysosporium*.

Antioxidant Activity

Figure 5 shows the result of DPPH radical scavenging activity for each fraction. All the fractions exhibited low scavenging activity compared to the positive control, quercetin (94.83 \pm 1.14%). A comparison between the fractions showed Fr. E exhibited the highest scavenging activity (29.52 \pm 1.32%), while Fr. A exhibited the lowest activity (2.71 \pm 1.56%). Subsequent ANOVA on the results showed a significant difference between fractions ($p < 0.01$), while Tukey HSD test indicated Fr. E exhibited significantly higher activity, compared to other fractions.

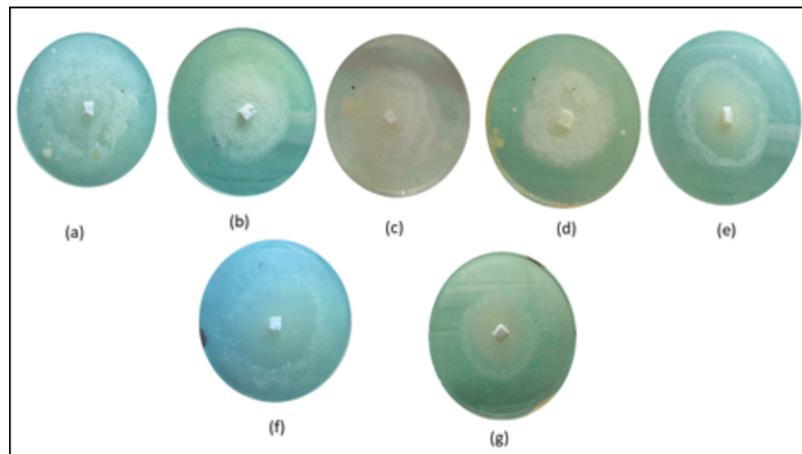


Figure 4. The growth rate appearance of EtOAc extract samples; Fr. A (a), Fr. B (b), Fr. C (c), Fr. D (d), Fr. E (e), control (f), lapachol (g)

Gambar 4. Kenampakan tingkat pertumbuhan antijamur dari sampel ekstrak EtOAc; Fr. A (a), Fr. B (b), Fr. C (c), Fr. D (d), Fr. E (e), control (f), lapachol (g)

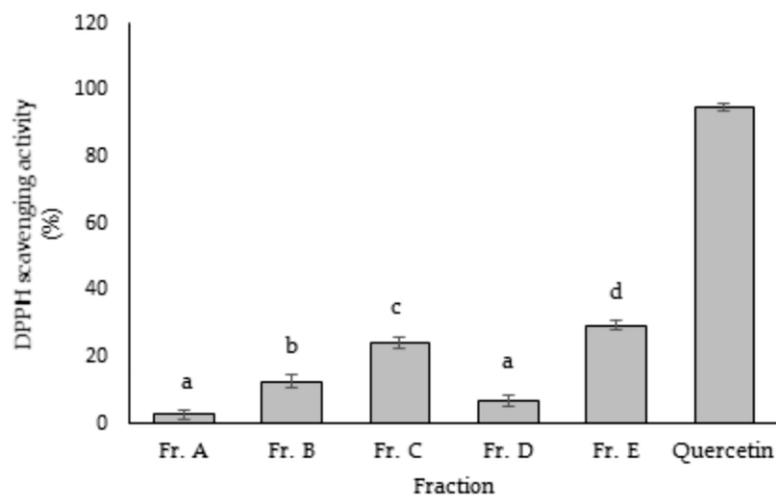


Figure 5. Antioxidant activity of Fr. A- Fr. E from ethyl acetate extracts. Different letters on the histogram (a, b, etc.) indicate significant difference of DPPH scavenging activity by Tukey HSD at 0.05 level

Gambar 5. Aktivitas antioksidan dari ekstrak etil asetat (Fr. A- Fr. E). Huruf yang berbeda pada histogram (a, b, dan seterusnya) menunjukkan perbedaan nyata nilai aktivitas DPPH pada uji lanjut Tukey HSD taraf nyata 0,05

Furthermore, of all the compounds detected in the all fractions, only α - terpineol was reported as antioxidant agent (Rodriguez et al. 2010). However, this compound was only detected in Fr. C, and a low concentration. For Fr. E, the antioxidant activity was lower compared to quercetin, and the higher antioxidant activity in Fr. E was possibly due to the high accumulation of steroid and triterpenoids. In the antioxidant assay, the DPPH solution with violet color was reduced to brown colored solution by the fraction samples (Fr. A- Fr. E), while quercetin reduced the solution to a yellow colored solution. The brown

colored product was suggested to be indicative of low antioxidant activity, while the yellow colored product indicated high antioxidant activity. Furthermore, the high antioxidant activity of quercetin was due to presence of catechol with two hydroxyl structure. The catechol hydroxyl group is responsible for neutralizing DPPH radicals (Bendary et al. 2013). However, for Fr. E, the hydroxyl structure occurred in 25-hydroxycholesterol and serrate-14-en-3 β ,21 β -diol. Due to high concentration of serrate-14-en-3 β ,21 β -diol in Fr. E, the compound was suggested to contain hydroxyl group acting as a DPPH neutralizer.

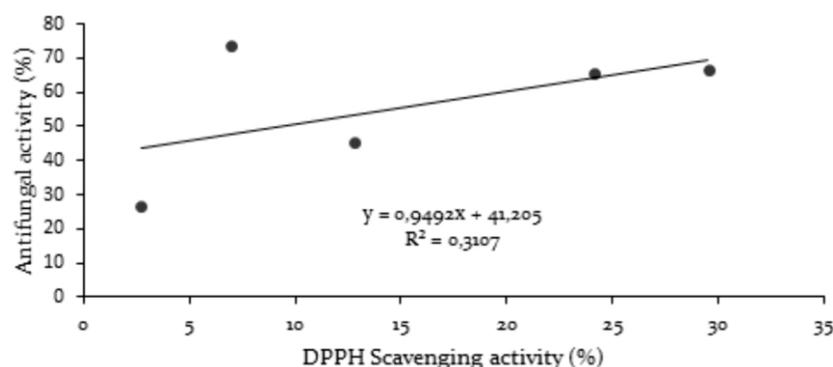


Figure 6. Correlation of antifungal activity and DPPH radical scavenging activity
Gambar 6. Korelasi antara nilai aktivitas antijamur dan aktivitas DPPH

Correlation between Antifungal Activity and Antioxidant Activity

According to the discussion above, both antioxidant and antifungal activity were attributed to the compounds with hydroxyl structure. The compounds 6 (25-hydroxycholesterol) and 9 (serrate-14-en-3 β ,21 β -diol) were indicated to be compounds contributing to antifungal and antioxidant activity in EtOAc extract of *P. merkusii* bark. This phenomenon was also reported by Mihara et al. (2005) in a study on *Acacia*, demonstrating a correlation between antifungal and antioxidant activity of the wood extract, where the antioxidant active compound was suspected to inhibit fungal growth by neutralizing the radicals produced by the fungi extracellular enzyme. Figure 6 also shows a correlation between antifungal and antioxidant activity. In this study, both activities showed low correlation ($R^2=0.3$), indicating the fungal growth inhibition of *P. merkusii* fractions were not attributed to the fractions' antioxidant activity. The low correlation shown in Figure 6 is also probably due to the presence of many compounds acting as stronger fungal inhibitors, rather than DPPH inhibitors. This is because the fractions of *P. merkusii* bark EtOAc extract are dominated by lipophilic compounds rather than phenolic or hydrophilic compounds with hydroxyl group. Furthermore, the low antioxidant values are also possibly attributed to the antioxidant method for lipophilic compounds, where using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical is

better for both hydrophilic and lipophilic compounds (Cano et al. 2000).

Conclusion

The fractionation of the ethyl acetate extract of *P. merkusii* bark produced five fractions (Fr. A- Fr. E). Based on the GC-MS analysis, mono-sesquiterpenoids, steroids, and hydrocarbons were detected. In addition, the antifungal activity of Fr. C-Fr. E, were higher, compared to Fr. A - Fr. B, while Fr. E exhibited the highest antioxidant activity. Meanwhile, the high content of steroids and triterpenoids in the isolated samples indicated the compounds were responsible for the fraction activities. The fractions' antifungal and antioxidant activities were discovered to have a low correlation and were also suggested to be contributed by the compounds with hydroxyl group. In the future, the re-isolation and purification of Fr. C-Fr. E is necessary to explain compounds acting as white-rot and DPPH inhibitors.

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