Methanol soluble extractives of *Pinus merkusii* bark and its antioxidant activity

Masendra, B A V Purba, and G Lukmandaru*

Department of Forest Products Technology, Faculty of Forestry, Gadjah Mada University, Yogyakarta, 55281, Indonesia

*Email: glukmandaru@ugm.ac.id

**Abstract.** The major function of bark in trees is to protect the living tissues. This is due to the chemical extractives in the bark such as polyphenols and terpenoids. In Indonesia, the bark of *Pinus merkusii* is of high economic value due to its use in the production of wood and oleoresins. Also, the presence of polyphenols in its bark has the potential of being used as antioxidant agents. Based on this, the aim of this study was to investigate antioxidant activity from the methanol extract of *P. merkusii* bark. This methanol extract of *P. merkusii* was subjected to gas chromatography mass spectrometric (GC-MS) method and antioxidant activity assay using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. The GC-MS analysis showed that the extract sample contained phenolics and sugars as minor compounds with fatty acids and alcohols as major components. The antioxidant activity (IC$_{50}$) of the sample was 1452.8 ± 208 µg/mL, which could be as a result of the presence of phenolic compounds, fatty acids and alcohols, as well as steroids and alkaloid in it.

1. Introduction

The major function of bark in plants is to protect the living tissues from extreme conditions such as low and high temperature, as well as from the attacks of microorganisms and herbivores. According to previous studies, the chemical compounds present in the bark are responsible for the protection ability [1-3].

In Indonesia, the plantation of *Pinus merkusii* occupies about 476,126 ha of forest area of Java. The needs for long fibers and oleoresins make this pine very important and it is the only species used from Pinaceae family. The production of oleoresins and wood from this species generally increase its demand. Moreover, the wood and bark of *P. merkusii* are very rich in both lipophilic and polyphenolic contents [4-7]. Hence, the use of this bark might be further developed for use in the pharmaceutical industries. A research also reported that the bark of pine could be used as antioxidant agents due to the presence of high polyphenolic compounds [8].

In addition, a study showed the difference between the ethanol extract of inner and outer bark of *P. merkusii* in terms of total phenolic, flavanol, and tannin-formaldehyde contents [7]. However, there is no study on the methanol (MeOH) soluble extract of *P. merkusii* and its antioxidant activity. Therefore, the purpose of this study was to investigate the constituents of MeOH fraction of *P. merkusii* bark and to evaluate its antioxidant activity.
2. Materials and Methods

2.1. Sample extraction

The bark sample of *P. merkusii* was obtained from Magelang, Central Java, Indonesia. About 1 kg of the bark sample was grind to powder (40 mesh) and extracted with *n*-hexane, ethyl acetate (EtOAc), and MeOH successively for 2 weeks at room temperature. The extract solution was then evaporated and weighed; the percentage of dry bark sample calculated.

2.2. Antioxidant activity

The antioxidant activity was measured with DPPH radical scavenging assay [9]. About 0.1 mL extract in methanol with different concentrations of 1000, 500, 250, and 125 μg/mL was reacted with 3 mL of 0.1 mM DPPH. This was incubated for 30 minutes at ambient temperature, after which the sample absorbance was read at 517 nm wavelength. The antioxidant activity was calculated through equation below:

\[
\text{Antioxidant activity (\%)} = \frac{100 \times (A_o - A_i)}{A_o}
\]

where *A*<sub>o</sub> is blank absorbance and *A*<sub>i</sub> is sample absorbance. In comparison, the standard of quercetin was assigned as the positive control. The value of antioxidant activity was shown in IC<sub>50</sub> (concentration of sample that inhibits 50% of DPPH radicals)

2.3. GC-MS analysis

To detect phenolic compounds using GC-MS, the MeOH extract sample was silylated according to Wijayanto *et al.* [4]. Briefly, 1 mg of MeOH extract was dissolved into 100 μL of TMCS (trimethylchlorosilane) and BSA (N, O-bis(trimethylsilyl) acetamide) (8:2, v/v). The solution was incubated at room temperature for 1 h. The solvent was then evaporated and the dry extract was again dissolved with 1 mL of MeOH. For analysis, 1 μL of sample was injected to GC-MS. The GCMS-QP2010 (Shimadzu, Kyoto, Japan) was used to analyze MeOH extract constituents. The specification of GC-MS column: Rtx-5 capillary column (30 × 0.25 mm inner diameter and 0.25 μm; GL Sciences, Tokyo, Japan). The condition of GC-MS as follows: column temperature from 70°C (1 min) to 295°C at 5°C/min; injection temperature of 200°C; detection temperature of 295°C; acquisition mass range of 50 amu to 700 amu using helium as the carrier gas. The mass spectra of sample were compared to NIST library. The quantification of detected compounds was calculated with relative peak method.

2.4. Total phenol content (TPC)

A modification method from Diouf *et al.* [10] was used for quantification of TPC. Briefly, A ten-fold dilution of Folin-Ciocalteu phenol reagent (2.5 mL) was mixed with 0.5 mL of sample (250 ppm). The mixture was stood for 2 min and to the solution, 2 mL of sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) was added, and the reaction mixture was again stood for 30 min at room temperature. Final solution was then read at 765 nm wavelength. The TPC results were expressed as gallic acid equivalents (mg GAE/g based on dry extract weight).

2.5. Total flavanol content (TVC)

The TVC was conducted according to Richard *et al.* [11] with vanillin HCl assay. Briefly, half milliliter of ethanol solution sample (250 ppm), 3 mL of vanillin reagent with 4% concentration in methanol, and 37% of HCl (1.5 mL) were reacted and the mixture was incubated for 15 min at ambient temperature. At 500 nm wavelength, the final sample absorbance was read. The TVC results were calculated by performing regression standard of (+) catechin (mg CE/g based on dry weight).

2.6. Tannin-formaldehyde (TF)

The TF was observed by literature of Humphreys [12]. The 2 g of bark sample was refluxed with distilled water in three times. The supernatant was filtered and the filtrate was cooled to 18°C. The solution was again diluted with distilled water until 500 mL. Further, 100 mL of the solution was used and reacted with 10 mL of formalin and 5 mL of HCl (37%). The mixture was then refluxed for 30 min. Final
equipment was performed by filtering and weighing the precipitate of sample. The tannin-formaldehyde is calculated with equation 2 as follows:

\[
\text{Tannin-formaldehyde (\%) = \left(\frac{4.425 \times A \times 10000}{W \times [100 – \text{sample moisture content}]}\right)
\]

(2)

where A is the weight of precipitate in gram and W is the weight of bark sample in gram.

2.7. Chemicals
Quercetin (≥ 95%), (+)-catechin (≥ 95%), DPPH, TMCS and BSA were purchased from Sigma-Aldrich (Germany).

3. Results and Discussion

3.1. Extractive yield and antioxidant activity
The extractive content of n-hexane has been reported in previous work [6], while that of EtOAc has not been reported. The MeOH extractive weighed 2.62 g or 0.26% of dried sample. Also, the antioxidant activity of MeOH soluble extract of P. merkusii bark is shown in Table 1. Based on the table, the antioxidant activity increased with increase in the sample concentration and the IC\(_{50}\) value calculated was 1452 ± 208 µg/mL (means ± standard deviation). This activity was low compared to positive control of quercetin (IC\(_{50}\) of 47.1 µg/mL).

The MeOH soluble extract yield from this study was lower than the ethanol soluble extractive of inner and outer bark of P. merkusii extracted with Soxhlet apparatus [7]. This could be as a result of difference in extraction methods. Similar result was also found in an earlier study on Thymelaea hirsuta [13]. Compared with the result of previous study on heartwood and knotwood of P. merkusii, the antioxidant activity of MeOH soluble extract from the bark in this study was higher than water soluble extracts but was lower than acetone soluble extracts [4]. Additionally, the MeOH extract of P. merkusii bark generally showed mild antioxidant activity in this study.

| Sample concentration (µg/mL) | DPPH Scavenging activity (%) | IC\(_{50}\) (µg/mL) |
|-----------------------------|-----------------------------|---------------------|
| 1000                        | 34.1 ± 4.7                  |                     |
| 500                         | 14.3 ± 3                    | 1452 ± 208          |
| 250                         | 4.7 ± 3.3                   |                     |
| 125                         | 1.6 ± 0.9                   |                     |

3.2. GCMS analysis
The GCMS analysis recorded the constituents of MeOH soluble extract of P. merkusii, as shown in Table 2. These constituents were categorized into phenolics, sugars, fatty acids and alcohols, steroids, and alkaloid. The fatty acids and alcohols group, composed of 53.68%, dominated the sample constituents while the predominant constituent was the steroids at 29.76%. Also, the concentration of lipophilic compounds extracted from the bark with n-hexane and ethyl acetate was high. The minor constituents detected by the GCMS method include alkaloid (10.68%), phenolics (2.79%) and sugars (1.91%). In these results, the phenolic constituent was detected in simple phenolic group, as shown in Table 2. In addition, the results showed that the phenolics and sugars detected were lower, possibly because some of these compounds were dissolved in EtOAc before MeOH extraction.

In the fatty acids and alcohols, methyl lignocerate and octacosoxy-trimethylsilane were the major compounds detected in the sample. However, the results were different from a previous study which involved analysing six different Pinus bark using the GCMS, without the detection of fatty acids [7]. In the case of steroids, the 25-hydroxycholesterol and stigmast-4-ene-3-one were the predominant constituents. These steroids were also found in bark of P. caribeae, P. oocarpa, and P. elliotii [5]. The alkaloid detected in higher amount from MeOH extract of P. merkusii was dodecahydro (1,2) oxazino
(2,3-b) isoquinoline. This is the first time this compound reported in the bark of *P. merkusii* as there were no detected previous study in relations to it.

The aromatic compound of 2-bromo-4-chloro-1-methoxybenzene was the dominant compound in the phenolics, while other monophenolics are from vanillin derivatives group. The presence of vanillin was also reported in *P. brutia* bark [14]. Lastly, the sugar compounds detected from the MeOH soluble extract include d-mannitol and beta-d mannofuranoside. This d-mannitol was also detected in bark of *P. elliotii, P. caribeae, P. montezumae*, and *P. insularis* [7].

**Table 2.** GC-MS derivative analysis of MeOH soluble extract from *P. merkusii*

| No | Ret. Time (min) | Constituents | Relative content (% of dried extract) | Similarity index (%) |
|----|----------------|--------------|--------------------------------------|---------------------|
| 1  | 21.2           | 2-Bromo-4-chloro-1-methoxybenzene | 0.83                                | 65                  |
| 2  | 21.3           | Vanillic acid hydrazide           | 0.76                                | 82                  |
| 3  | 26.4           | Vanillin, acetate                 | 0.69                                | 71                  |
| 4  | 26.9           | Vanillyl methyl ketone            | 0.51                                | 74                  |
|    |                | **Phenolics**                      | 2.79                                |                     |
| 5  | 21.4           | D-Mannitol                        | 0.78                                | 89                  |
| 6  | 22.8           | beta-d-Mannofuranoside,           | 1.13                                | 80                  |
|    |                | **Sugars**                         | 1.91                                |                     |
| 7  | 26.6           | 9-Hexadecen-1-ol                   | 2.37                                | 97                  |
| 8  | 39.0           | Methyl lignocerate                 | 13.15                               | 90                  |
| 9  | 39.4           | trans-13-Octadecenoic acid         | 0.65                                | 89                  |
| 10 | 39.5           | 3-Heptadecanol                     | 0.77                                | 76                  |
| 11 | 39.8           | Ethyl docosanoate                  | 2.92                                | 88                  |
| 12 | 40.0           | Heptadecyl acetate                | 3.63                                | 95                  |
| 13 | 40.2           | Methyl pentacosanoate              | 0.47                                | 79                  |
| 14 | 40.7           | 3-Hexadecanol                      | 1.93                                | 81                  |
| 15 | 41.1           | Ergost-5-en-3-yl acetate           | 0.65                                | 65                  |
| 16 | 41.4           | Methyl hexacosanoate               | 7.52                                | 95                  |
| 17 | 42.0           | Methyl heptadecyl ketone           | 3.87                                | 82                  |
| 18 | 43.4           | Epoxy-linalooloxide                | 1.71                                | 78                  |
| 19 | 43.6           | Methyl palmitate, (2,2-dimethyl-1,3-dioxolan-4-yl) | 3.66 | 70 |
| 20 | 47.4           | Octacosoxy-trimethylsilane         | 10.38                               | 70                  |
|    |                | **Fatty acids and alcohols**       | 53.68                               |                     |
| 21 | 42.2           | Dibromostigmasterol acetate        | 3.64                                | 80                  |
| 22 | 43.7           | Stigmast-5-en-3-ol, olate          | 1.61                                | 75                  |
| 23 | 44.0           | Cholesta-4,6-dien-3-ol             | 4.91                                | 81                  |
| 24 | 44.2           | beta-Sitosterol                    | 0.72                                | 65                  |
| 25 | 44.3           | Stigmast-4-ene-3-one               | 7.78                                | 88                  |
| 26 | 48.9           | gamma-Sitosterol                   | 3.89                                | 82                  |
| 27 | 49.1           | 25-Hydroxycholesterol              | 7.21                                | 72                  |
|    |                | **Steroids**                       | 29.76                               |                     |
| 28 | 50.7           | Dodecahydro (1,2) oxazino (2,3-b) isoquinoline | 10.68 | 65 |
| 29 |                | **Alkaloid**                       | 10.68                               | 65                  |
3.3. TPC, TVC, and TF

The values of phenols contents of inner and outer bark of *P. merkusii* are shown in Table 3, as reported in a previous study by Masendra et al. [7]. Both the inner and outer bark contained higher TPC, while higher amount of TVC was observed in the inner bark. Then, the TF was found in outer bark sample. Based on a previous study, phenol contents were also found in the bark of *P. thunbergii*, *P. banksiana*, *P. radiata*, and *P. koraiensis* [8].

| Phenol contents | Inner bark | Outer bark |
|-----------------|------------|------------|
| TPC             | 421.4      | 486.5      |
| TVC             | 367.4      | 55.2       |
| TF              | -          | 0.4        |

(·): not determined TPC: mg GAE/ g extract sample; TVC: mg CE/ g extract sample; TF: % of dried bark sample

3.4. Correlation between antioxidants and related compounds

The phenolics components of the MeOH soluble extract of this bark sample could be responsible for its antioxidant activity. However, these phenolic constituents were present in lower amount compared with other compounds. Hence, it was suggested that other major compounds such as fatty acids and alcohols played a major role in reducing the DPPH into DPPH-H. According to a previous literature, polyunsaturated fatty acids could act as antioxidant [15]. Additionally, other compounds capable of contributing to the antioxidant property are steroids and alkaloids. A previous paper showed that sterols from Turkish olive oils are potent for DPPH inhibitor [16]. Furthermore, the higher alkaloid such as quinoline derivatives could also act as antioxidant agents [17].

In comparison with phenols content, higher TPC was observed in the bark of *P. merkusii*. This suggested that TPC had a greater effect in inhibiting DPPH. However, the high concentration of TVC in the inner bark and TF in the outer bark indicated that flavan-3-ol structure (C4-C8 bonds) plays a major role in increasing the bark’s antioxidant activity. These findings were in line with the results of the study conducted by Ku et al. [8] involving the bark of the following species; *P. densiflora*, *P. thunbergii*, *P. banksiana*, *P. contorta*, *P. rigida*, *P. taeda*, *P. serotina*, *P. radiata*, *P. koraiensis*, *P. parviflora*, and *P. rigida x taeda*.

4. Conclusion

Based on the findings, the antioxidant activity (IC$_{50}$) of the bark sample was 1452.8 ± 208 µg/mL, categorized under mild activity. Analysis of the bark sample through GCMS detected fatty acids and alcohols as the dominant compounds while phenolics and sugars were the minor compounds. The value of the antioxidant activity in the sample, as reported in this study, was attributed to the presence of phenolics and other compounds such as steroids, alkaloid, as well as fatty acids and alcohols.

References

[1] Wittstock U and Gershenzon J 2002 Constitutive plant toxins and their role in defense against herbivores and pathogens *Curr. Opin. Plant. Biol.* 5(4) 300-7
[2] Alfredsen G, Solheim H, and Slimestad R 2008 Antifungal effect of bark extracts from some European tree species *Eur. J. For. Res.* 127 387-93
[3] Seki K, Orihashi K, Sato M, Kishino M, and Saito N. 2012. Accumulation of constitutive diterpenoids in the rhytidome and secondary phloem of the branch bark of *Larix gmelinii* Var. *Japonica J. Wood Sci.* 58 437-45
[4] Wijayanto A, Dumarçay S, Gérardin-Charbonnier C, Sari RK, Syafii W, and Gérardin P 2015 Phenolic and lipophilic extractives in *Pinus merkusii* Jungh. et de Vries knots and stemwood *Ind. Crops. Prod.* 69 466-71
[5] Masendra, Ashitani T, Takahashi K, and Lukmandaru G 2018 Lipophilic extractives of inner and outer barks from six different Pinus species grown in Indonesia. J. For. Res. 29(5) 1329-36
[6] Masendra, Ashitani T, Takahashi K, and Lukmandaru G 2018 Triterpenoids and steroids from the bark of Pinus merkusii (Pinaceae) Bioresources 13(3) 6160-70
[7] Masendra, Ashitani T, Takahashi K, Susanto M, and Lukmandaru G 2019 Hydrophilic extracts of the bark of six Pinus species J. Korean Wood Sci. Technol. 47(1) 80-9
[8] Ku CS, Jang JP, and Mun SP. 2007. Exploitation of polyphenol-rich pine barks for antioxidant activity J. Wood Sci. 53 524-8
[9] Baba SA and Malik SA 2015 Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of Arisaema jacquemontii Blume J. Taibah. Univ. Sci. 9(4) 449-54
[10] Diouf PN, Stevanovic T, and Cloutier A 2009 Antioxidant properties and polyphenol contents of Trembling aspen bark extracts Wood Sci. Technol. 43(4) 457-70
[11] Richard T, Broadhurst B, and Willian JT 1978 Analysis of condensed tannins using acidified vanillin J. Sci. Food Agric. 29 788-94
[12] Humphreys FR 1956 Determination of tannin-formaldehyde in bark Soc. Leather Trade Chemist J. 40 147-9
[13] Yahyaoui M, Ghazouani N, Sifaoui I, and Abderrabba M 2017 Comparison of the effect of various extraction methods on the phytochemical composition and antioxidant activity of Thymelaea hirsuta L. aerial parts in Tunisia. Biosci. Biotechnol. Res. Asia 14(3) 997-1007
[14] Kivrak I, Kivrak S, Harmandar M, and Cetintas Y 2013 Phenolic compounds of Pinus brutia Ten.: chemical investigation and quantitative analysis using an ultra-performance liquid chromatography tandem mass spectrometry with electrospray ionization source Rec. Nat. Prod. 7(4) 313-319
[15] Richard D, Kefi K, Barbe U, Bausero P, and Visioli F 2008 Polyunsaturated fatty acids as antioxidants Pharmacol. Res. 451-5
[16] Yorulmaz HO, and Konuskan, DB 2017 Antioxidant activity, sterol and fatty acid compositions of Turkish olive oils as an indicator of variety and ripening degree J. Food Sci. Technol. 54(12) 4067-77
[17] Püsküllü MO, Tekiner B, and Suzen, S 2013 Recent studies of antioxidant quinoline derivatives Mini Rev. Med. Chem. 13 365-72

Acknowledgement
We thank to BIDIK MISI Scholarship (Indonesia) for supporting this research, and Mr. Sukmono Edwi Susanto (Perum Perhutani) for providing the bark sample of P. merkusii.