Polyphenol contents and free radical scavenging activity of *Pinus merkusii* cone

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Abstract. This study aimed to investigate the polyphenol contents from the *Pinus merkusii* cone extract and their antioxidant activity. The cone powder (40 mesh) was extracted with *n*-hexane, ethyl acetate, and methanol successively for six hours. The cone of *n*-hexane, ethyl acetate, and methanol soluble extracts was subjected to total tannin content, total phenol content, total flavonoid content, and antioxidant measurements. The polyphenol contents were measured through the colorimetric method, while antioxidant activity was observed by inhibition of the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. The results showed that total tannin content, total phenol content, total flavonoid content, and antioxidant activity of methanol soluble extract was higher than ethyl acetate and *n*-hexane soluble extracts. In this study, the high antioxidant activity of methanol soluble extract (IC₅₀ of 196.73±13.93 ppm) might due to the high concentration of total phenol content (545.38±54.07 mg GAE/g sample). It was suggested that the *P. merkusii* cone is potential as the antioxidant source.

1. Introduction

In Indonesia, the site of forest pine is calculated for about 600,000 ha especially on Java Island. The forest pine (*Pinus merkusii*) is managed by the Perhutani enterprise to obtain oleoresins and the wood that is utilized for the use of long fiber products. However, in its living process, the pine also produces a higher number of cones for generative reproduction. Once it is finished, unlike the wood, the dried cone of *P. merkusii* is not utilized yet. In previous works, the pine cone was reported to contain hydrophilic, and lipophilic extractive [1,2,3]. Therefore, the utilization of *P. merkusii* cone as the pharmaceutical industry is estimated in greater potency such as for antioxidant sources.

The antioxidant agent is a material that can inhibit radical scavenging activity such as DPPH (1,1-diphenyl-2-picrylhydrazyl). The inhibition of DPPH is initiated by the change of its color from dark purple to light color or yellow. One of the materials for DPPH inhibitors is phenolic compounds, namely, gallic acid and catechin [4]. The ability of phenolic compounds to inhibit DPPH is due to the presence of hydroxyl and double bonds on their benzene ring. The hydroxyl structure is sensitive and is easily reacted with DPPH. The phenolic compounds are produced by trees or plants as their secondary metabolite with the shikimic acid pathway as its synthesis process. In previous work, the plant's part of *Pinus merkusii* was reported to contain phenolic compounds from its bark and wood [5,6,7]. However,
the information of phenolic compounds from their cone is not investigated yet. Thus, this study aims to investigate phenol contents of *P. merkusii* cone extract together with DPPH activity.

2. **Material and method**

2.1. **Cone collection and extraction**
The cone of *P. merkusii* was obtained from Temanggung, Central Java, Indonesia. The cone was ground to obtain a powder of 40 mesh. The dry cone (53.5 g) was extracted in a Soxhlet apparatus for 6 h using *n*-hexane, ethyl acetate (EtOAc), and methanol (MeOH), successively. The extracts solution were further evaporated and the dried extract was weighed as result (percentage in dry sample).

2.2. **Total tannin content (TTC)**
To observe TTC in the pine cone extracts, the literature of Padmaja [8] was referred. To the 0.1 ml sample solution (1000 µg/ml), a 7.5 ml of distilled water was added for dilution. The mixture sample then was reacted with Folin Denis (0.5 ml) and 35% of sodium carbonate (1 ml). The performance of 30 min incubation was stood to the sample before observing the sample absorbance at 760 nm read by a visible spectrophotometer (WPA S800+). The curve calibration of tannic acid was made to calculate TTC (mg tannic acid equivalent/g sample).

2.3. **Total phenolic content (TPC)**
The measurement of TPC was conducted through the reaction of 500 µl of extract solution (1000 µg/ml) with a ten-fold dilution of Folin-Ciocalteu (2.5 ml). The mixture was maintained for 2 min at room temperature. After the incubation, to the solution sample the addition of 2 ml sodium carbonate (7.5% aqueous) was conducted. Once the addition of sodium carbonate was done, the sample was continued for 30 min incubation. The wavelength of 765 nm was used to read the absorbance solution using a visible spectrophotometer (WPA S800+) and the curve calibration of gallic acid was made to measure TPC in the sample [9].

2.4. **Total flavonoid content (TFC)**
The presence of TFC in the pine extracts was observed by AlCl₃ method [10]. An extract sample (2 ml and 1000 µg/ml concentration) was reacted with 2% of AlCl₃·6H₂O solution (2 ml). The mixture was stood at 20 °C and at 415 nm wavelength the absorbance value was measured using a visible spectrophotometer (WPA S800+). Standard of flavonoid of quercetin was chosen to express the TFC unit (mg quercetin equivalent/g sample).

2.5. **Antioxidant activity**
The antioxidant measurement was conducted based on literature [11]. Briefly, the mixture of 0.1 ml of sample and 3 ml of DPPH (0.1 mM) was maintained for 30 min. The sample solution then was read at 517 nm using a UV-Vis spectrometer. The antioxidant activity was evaluated in IC₅₀ values and the experiment was done in three replications. In this study, phenolic of quercetin used for positive control. The inhibition of DPPH is calculated through equation 1 as follows:

\[
\text{Antioxidant} = \frac{(A_o - A_1)}{A_o} \times 100\% \quad (1)
\]

Where Ao is the absorbance of blank and A1 is sample.

2.6. **Gas chromatography-mass spectra**
The silylated method [12] was used to investigate the phenolics of *P. merkusii* cone. Briefly, 1 mg sample was dissolved with pyridine (50 µl) and then 99 µl of BSA and 1 µl of TMCS were added. The sample was heated for 30 min at 100 °C. After heating, the sample was conditioned at ambient
temperature for 20 min and then 1 ml of MeOH was delivered to the sample solution. The sample (1 µl) was injected into the GC-MS machine and data was obtained from a GCMS-QP 2010 (Shimadzu, Japan). The capillary column of Rtx-5MS (length of 30 m, diameter of 0.25 mm, and internal diameter of 0.25 µm; GL Sciences, Tokyo, Japan). The detection temperature was made at 285 °C with column temperature from 70 °C (2 min) to 290 °C at 5 °C/min. The injection temperature was set at 200 °C with helium as the gas carrier. For qualitative comparison, the mass spectra from NIST11 library was used.

2.7. Chemicals
Tannic acid, gallic acid, and quercetin, pyridine, N, O-Bis(trimethylsilyl)acetamide or BSA, and TMCS (trimethylchlorosilane) were obtained from Sigma-Aldrich (Germany).

2.8. Statistical data analysis
Analysis of variance (ANOVA) was used to express the significance of the factor of solvent at 95% confidence level. Data with a significant difference were analyzed with Tukey-HSD. The statistical analysis was done with SPSS software (IBM, USA).

3. Result and discussion
3.1. Extractive and Phenol contents
The extraction of P. merkusii cone through n-hexane, EtOAc, and MeOH solvent yielded 3.1%, 1.9%, and 6.3%, respectively. Those yields were similar range from the cone of P. halepensis, P. pinea, P. sylvestris, P. nigra, P. brutia [1]. Further comparison was also made with the bark and wood of P. merkusii, where the pine cone extractive from the present study was in similar results [5, 6, 13, 14]. The phenol content of the P. merkusii cone is shown in Figure 1. The phenol content was dominated by TPC followed by TTC and TFC. This indicates that n-hexane soluble extract is dominated by non-phenolic terpenes. This was similar to previous work reported by Kilic et al. [1], that pine cone from several Pinus species was confirmed containing terpenes.

In Figure 1, the MeOH soluble extract polyphenol contents were higher than n-hexane and EtOAc soluble extracts. The greatest total phenolic and tannin content was observed in MeOH extract with the result of 545.38±54.07 mg GAE/g and 184.84±1.36 mg TAE/g, respectively, indicating that MeOH solvent is more polar than n-hexane and EtOAc solvent, and highly extracted phenolic compounds from the P. merkusii cone. In comparison, the phenol content of P. merkusii cone was greater than P. nigra and P. brutia cone [15]. Compared to its bark [6], the phenol content of P. merkusii was also in the high range. It is assumed that the higher extractive in the cone is due to the function of this part in the tree as generative reproduction as well as for food storage.

Figure 1. Phenol content of P. merkusii cone; TTC (total tannin content in mg TAE/g), TPC (total phenol content in mg GAE/g), and TFC (total flavonoid content in mg QE/g); The same letters are not statistically different at p < 0.05 by Tukey test (p<0.01)
3.2. Antioxidant activity

The DPPH inhibition of n-hexane, EtOAc, and MeOH soluble extracts of _P. merkusii_ cone was displayed in Figure 2. The n-hexane and EtOAc soluble extracts exhibited lower inhibition than MeOH soluble extract. The n-hexane and EtOAc soluble extracts were ten times lower than MeOH soluble extract. However, compared to quercetin, the extract of _P. merkusii_ cone was lower activity than quercetin. The antioxidant activity of _P. merkusii_ cone of MeOH soluble extract in this study was also lower than _P. brutia_ and _P. nigra_ [15]. Furthermore, the antioxidant of the non-polar compound in n-hexane extract of _P. merkusii_ also was in low activity compared to _P. armandii_ cone oil [16].

![Figure 2. Antioxidant activity of _P. merkusii_ cone extract](image)

In correlation to phenols content, the lowest IC$_{50}$ value of MeOH extract from the cone of _P. merkusii_ is might correlate to high total phenolic content (In Figure 3). The phenol contents and antioxidant activity showed in high correlation ($R^2$>0.9), especially in the total flavonoid contents. Thus, it is assumed that phenol contents are responsible for inhibiting DPPH. Moreover, as the MeOH extract of _P. merkusii_ cone showed as the most inhibiting extract, the MeOH extract was injected into GC-MS to investigate its phenolic compounds. Figure 4 demonstrated that mono and phenolic compounds were detected from the cone of _P. merkusii_ of MeOH soluble extract. The detection of phloroglucinol and (+)-catechin in MeOH extract of _P. merkusii_ cone might involve inhibiting free radical or DPPH. This was reported that phloroglucinol [17] and (+)-catechin [4] are good phenolics as an antioxidant agent.

![Figure 3. Correlation between antioxidant activity and phenol content; TTC (grey circle), TPC (black circle), TFC (white circle)](image)
Figure 4. GC-MS chromatogram of the MeOH extract from P. merkusii cone; phloroglucinol and its isomer (a) and (+)-catechin (b)

4. Conclusion
The determination of P. merkusii cone polyphenols is dominated by TPC, followed by TTC and TFC. The antioxidant investigation on the P. merkusii cone showed the MeOH extract as the highest value. The greatest antioxidant activity of P. merkusii cone MeOH extract correlates with the high total phenolic content of MeOH soluble extract especially phloroglucinol and (+)-catechin.

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