



THE BIOACTIVE OF *PINUS MERKUSII* NEEDLE AND BARK EXTRACT AS ANTIOXIDANT AND ANTIAGING

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ABSTRACT

Pinus merkusii is a native pine species to Southeast Asia and has used as an oleoresins source and raw material for pulp and paper industries. This plant also possesses several biological activities, such as anti-inflammatory and larvicidal activity. This study aims to evaluate the antioxidant and antiaging activity of *P. merkusii* needle and bark. The qualitative phytochemical screening was used to evaluate the presence of secondary metabolites compounds. DPPH (2,2-diphenyl-1-picryl-hydrazyl) methods evaluated the antioxidant activity, and an anti-tyrosinase assay was used to evaluate the antiaging activity. Phytochemical analysis showed flavonoids, phenols, alkaloids, tannins, and terpenoids in both extracts. Bark extract showed the presence of saponins and triterpenoids, while needle extract possesses steroids. The antioxidant activity (IC₅₀) of *P. merkusii* bark extract was 59.32 ± 1.74 µg/mL, stronger than needle extract (68.67 ± 1.47 µg/mL). Also, the bark extract showed higher inhibitory activity of tyrosinase (IC₅₀) 74.97 ± 1.54 µg/mL than needle extract (96.08 ± 1.77 µg/mL). From this investigation, *P. merkusii* bark extracts appeared to have more potential as a natural source of antioxidants and antiaging and might be beneficial in these subjects.

Keywords: *Pinus merkusi*; Antioxidant activity; Anti-aging; Tyrosinase Inhibition

INTRODUCTION

Skin aging has a complex biochemical process where collagen and elastin degradation occurring in the epidermal and dermal layer, which connect to the extracellular matrix (ECM). Matrix metalloproteinases (MMPS) is the enzyme that involved in the degradation of ECM [1]. Degradation of extracellular matrix (ECM) contributes to the skin losing its tensile,

where MMPs contribute to establishing the wrinkle [2]. Exposure to UV radiation is known as extrinsic factors that conducted the activation of tyrosinase, elastase and collagenase, resulting in skin aging, wrinkle formation, and melanin production [3], [4].

Oxidative stress plays a main role in the aging process since it is harmful to the skin and ROS overproduction [5]. Oxidative stress resulted from the unbalance of ROS and antioxidants [6]. ROS formation by UV

radiation exposure could interact with proteins, lipid, and DNA and causing aging-related disorder [7]. Excess production of ROS will certainly lead to DNA mutations in elastic fiber proteins that cause a decrease in collagen. This phenomenon was causing the formation of wrinkles and skin laxity [8]. The strategies to prevent skin aging is by maintaining antioxidant homeostasis [9]. ROS are removed from the body by the defence system of antioxidants [10] since the antioxidants possess bioactive components that have been proposed for the prevention of aging [11].

Antioxidants can scavenge free radicals such as reactive oxygen species (ROS), including superoxide anion, peroxides, and singlet oxygen that are harmful to humans [12]. In the process of exogenous aging, constant skin exposure to it triggers fibroblasts to produce ROS. This reaction causes a structural change in extracellular matrix (ECM), such as collagen, proteoglycan, elastin, and fibronectin [13], [14]. The degradation of ECM connected with skin aging and collated with the increasing activity of certain enzymes involved in skin aging [15], [16]. The role of antioxidants in preventing skin aging is essential since these compounds can neutralize free radicals by donating or accepting an electron to complete the unpaired molecules [17]. Moreover, antioxidant to prevent skin aging is also supported by its anti-inflammatory properties [17].

Pinus merkusii is the native species to Southeast Asia known as Sumatra pine, located in Indonesia, mainly in northern Sumatra; Aceh, Tapanuli, and Kerinci [18],

[19]. *P. merkusii* mostly found in acidic and poor soils with an elevation between 800 and 200 m above sea level [18]. It is known that Indonesia is among the top three countries for the production of *P. merkusii* resin [20]. This pine has been used as a natural source for oleoresins to produce gum rosin and turpentine, while the wood is used as raw material for pulp and paper industries [21]. In the pulp industry, pine bark is mostly left discarded or used for fuel. The discarded pine bark left in large amount since pine bark accounts for 10-15% of the whole pine tree [22].

Several studies revealed that pine has high therapeutic value and has potential as a drug in the future due to its bioactivity, such as antioxidant, antimicrobial, antifungal, and anti-inflammatory [23]. *P. pinaster*, as the common pine that could be found in several places since it was easy to grow was reported to have anti-inflammatory, antioxidant, and wound healing activity in its essential oil [24].

Previous studies demonstrated that phenolic compounds such as matairesinol and nortrachelogenin and flavonoid compound such as pinocembrin are found in *P. merkusii* bark extract [19]. This is also confirmed by the findings [25] who identified flavonoid compounds from *P. merkusii* bark extract. The antioxidant, anti-inflammatory, and antifungal properties of flavonoid are considered essential in pharmaceutical and cosmetic applications [26]. For example, Pinocembrin, a flavonoid, has been reported to have the capability of absorbing UV rays, which enhance the possibility of its usage as a sunscreen in photoprotection [27]. Pinocembrin was also used as an antifungal

since it could inhibit the mycelial growth of *Penicillium italicum* on the skin (peng). Matairesinol, a lignan from the phenolic compound, were also reported to have potential as antiaging agents [28].

Although the information about *P. merkusii* is available, the information about the bioactivity of barks and needles is still very limited. Therefore, we investigated the antioxidant activity and evaluated the antiaging capacity of bark and needle extract of *P. merkusii*. We performed phytochemical screening to identify substances contained in the extracts.

METHODS

1. Materials

a. Raw Materials

Pinus merkusii needle and bark samples were collected from Pine Recreation Forest in Mount Tangkuban Perahu area, Jawa Barat, Indonesia (-6.7802740, 107.6464412) and was authenticated by Botanist from the Biology Department, Bandung Institute of Technology, Bandung, Indonesia.

b. Chemicals

Iron(III) chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), magnesium powder, mercury(II) chloride, potassium iodide, iodine, bismuth(III) nitrate, acetic anhydride, tyrosinase, L-tyrosine were purchased from Sigma-Aldrich, USA. Phosphate buffer, chloroform, hydrochloric acid, sulfuric acid, amyl alcohol, and ethanol were obtained from Smart-Lab, Indonesia.

2. Research Methods

a. Preparation and Sample Extraction

The preparation and extraction of samples were determined by the following previously method with modification [29]. A botanist from Bogor Botanical Garden authenticated *P. merkusii* needle and bark were collected from Pine Recreation Forest in Mount Tangkuban Perahu area, Jawa Barat, Indonesia and. A 2,700 g barks were cut then washed along with 2,600 g needles separately. Both samples dried at 50°C for two days and ground into powder, resulted in 1,200 g of needles and 2,150 g of barks. Both samples were extracted with 4000 mL of 96% ethanol (v/v) and submitted to maceration for 24 h at room temperature. Subsequently, the mixtures were filtered, and the extraction process was repeated twice. All of the extracts were concentrated at 50°C using a rotary evaporator and obtained.

b. Phytochemical Screening

The filtrated of *P. merkusii* needle and bark was evaluated by qualitative assay of common plant secondary metabolites. The screening was carried out for flavonoids, alkaloids, tannins [30], saponins [31], phenols [32], steroid, terpenoids [33], and triterpenoid [34]. The changing of color, frothing, or precipitate formation was used for the test response.

c. Antioxidant Capacity Analysis

The antioxidant capacity of extracts was investigated using a DPPH assay. This assay was followed the previous method [35]. A 200 μ L of 7 μ mol DPPH was added into 50 μ L of crude extracts. The mixtures were

incubated for 30 min at room temperature and measured at 517 nm. A 250 μ L of DPPH was used as a negative control, and 250 μ L of DMSO was used as the blank solution. The values of scavenging activity were calculated as formula below:

$$\text{Scavenging activity (\%)} = \frac{(A_c - A_s)}{A_c} \times 100\%$$

A_c = negative control absorbance (without sample)

A_s = sample absorbance

The IC_{50} value was also determined as the sample concentration (μ g/mL) required to inhibit 50% of the activity (IC_{50}) calculated from a dose-response curve using GraphPad software (San Diego, CA, USA).

d. Anti-tyrosinase Activity Assay

The crude extracts were dissolved into various concentrations (3.13; 6.25; 12.5; 25; 50; 100 μ g/mL) and 20 μ L extract was added into 96-well plate. Subsequently, 20 μ L of mushroom tyrosinase (500 U/mL) were added into the well, followed by 140 μ L of 20 mM phosphate buffer pH 6.8. The mixtures were incubated for 15 min at room temperature. A 20 μ L of 0.85 mM L-tyrosinase to each well and incubated for 10 min at 25°C and the enzymatic activity was measured at 470 nm [7]. The value of enzymatic inhibition was calculated then present as median inhibitory concentration (IC_{50}) by GraphPad software (San Diego, CA, USA).

$$\text{Inhibition (\%)} = \frac{(A_c - A_s)}{A_c} \times 100\%$$

A_c = negative control absorbance (without sample)

A_s = sample absorbance

RESULTS AND DISCUSSION

1. Sample Extraction

Before analyzing the bioactivity of the pine needles and bark, the extracts from the samples were first prepared through the maceration process. The yield of *P.merkusii* needle and bark extract was 56.82 g and 72.35 g, respectively.

2. Phytochemical Screening

Secondary metabolites are chemical compounds that possess various biological activity [36], which provide the basis for using herbs as a traditional remedy [37]. Phytochemical analysis is one technique for preliminary identification of chemical content in the plant extract that serves important biological roles as defensive compounds or chemical messengers [38].

Table 1. Secondary metabolites present in *P.merkusii* needle and bark extracts

Compounds	Needle extract	Bark extract
Alkaloids	(+)	(+)
Flavonoids	(+)	(+)
Phenols	(+)	(+)
Saponins	(-)	(+)
Steroids	(+)	(-)
Triterpenoids	(-)	(+)
Tannins	(+)	(+)
Terpenoids	(+)	(+)

+ = Present, - = Absent

Table 1 shows the preliminary investigation of secondary metabolites in *P.merkusii* needles and barks extracts. This screening test was carried out to provide an overview of the class of compounds in the 96% ethanol extract of pine needle and bark.

The screening test revealed alkaloids, flavonoids, phenolics, saponins, triterpenoids, tannins, and terpenoid. These results indicate that the bark and needle of the *P. merkusii* have the potential as an antioxidant because these compounds, in general, can have antioxidant properties [39]. Also, saponins and polyphenols have proven to be able to enhance anti-tyrosinase activity [40].

The ethanol extract of needles and barks contained the same compounds, except for needle extract that contained steroids without triterpenoids and vice versa for bark extract. The difference in the content of these secondary metabolites is inseparable from plant organ observation since these compounds were accumulated at various stages of plant organ growth. Their accumulation rates are different at each stage of the growth [41]. Both of these two extracts contained polyphenols compounds such as flavonoids and phenols known to have high antioxidant activity due to their capacity to inhibit reactive oxygen species [42]. In previous research, [25] found that ethyl acetate extract of bark of *P.merkusii* contained flavonoid compound while tannins were identified in ethanolic-extract of *P. merkusii* barks [43].

3. Antioxidant Activity

Since both extract potentially has antioxidant properties, the antioxidant activity of needles and barks extract was investigated using the extract's ability in inhibiting DPPH radical. The values of the scavenging activity stated in IC_{50} for each extract were shown in Figure 1.

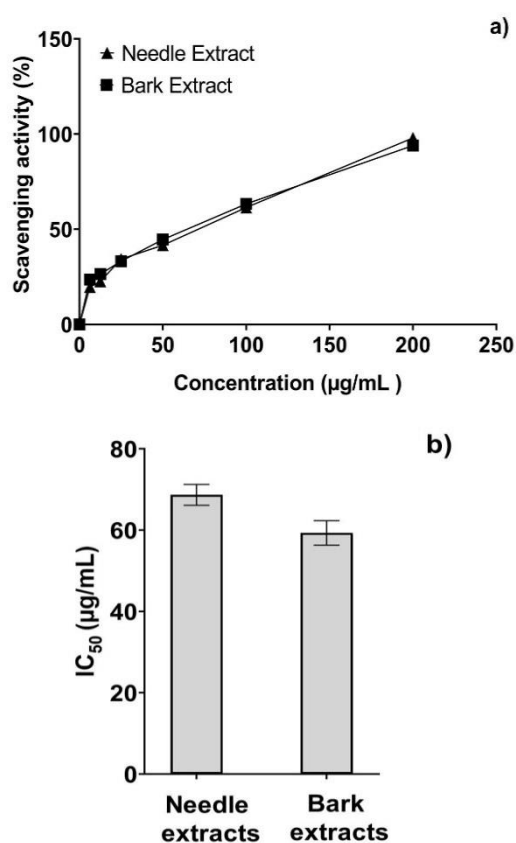


Figure 1. Scavenging activity of extracts in various concentration a) and scavenging activity in IC_{50} value b).

In Figure 1a, the DPPH scavenging activity of pine needles and bark extract showed the same trend in dose-dependent manners. At the 200 µg/mL extract concentration, all of the extracts showed high scavenging activity with 97,98% and 93,93% value for needle and bark extracts, respectively. In Figure 1b, the needle extracts showed 68.67 ± 1.47 µg/mL while bark extract showed 59.32 ± 1.74 µg/mL IC_{50} value. It could be seen that the antioxidant properties of bark extract stronger than needle extract.

The capacity of natural extracts to scavenge free radical is one of the most researched bioactivities of natural compounds, as the ability to prevent oxidative stress and several diseases. This capacity depends on

the composition of bio compound in the plant extracts, such as flavonoids and phenolic and their ability to neutralize free radical through several mechanisms [6]. The previous study showed antioxidant activity (IC_{50} value) of pine bark extract from *P. pinaster* was 100.1 $\mu\text{g/mL}$ [44]. The antioxidant capacity of pine needle extract was also studied previously by [45] using *P. densiflora* species and resulted in 373.70 $\mu\text{g/mL}$ in 100% ethanol. In contrast, ethanolic extract of *P. thunbergii* bark had 87.5% inhibition in [46]. Compared with those results, extracts of our *P. merkusii* needle and bark showed stronger activity. It means that the needle and bark of *P. merkusii* pine have potential as a source of natural antioxidants.

4. Anti-tyrosinase assay

Since the needle and bark extract of *P. merkusii* showed several secondary metabolites related to antioxidants, Tyrosinase inhibition activity of ethanol extract of *P. merkusii* needle and bark were analyzed using the dopachrome method with L-DOPA as the substrate. The anti-tyrosinase activity of both extracts are depicted in Figure 2 below;

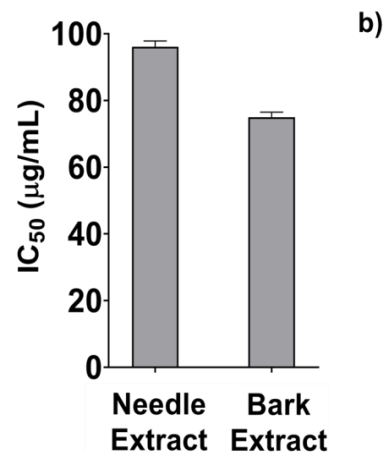
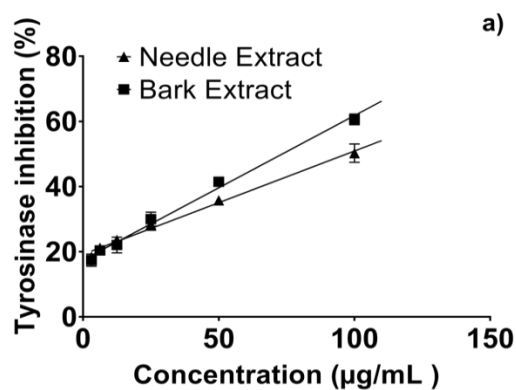


Figure 2. Tyrosinase inhibition of *P. merkusii* needle and bark extracts in various concentration a) and the IC_{50} value b).

The enzyme inhibitor is an agent capable of reducing enzymatic reactions, such as the melanogenesis pathway [47] and food browning [48]. The capacity of *P. Merkusii* needle and bark extract in inhibiting tyrosinase shown in Figure 2a) as increased of extract concentrations. In the Figure 2b), the study revealed that bark extract inhibited tyrosinase with IC_{50} value $74.97 \pm 1.54 \mu\text{g/mL}$ (60.64% inhibition) while needle extract $96.08 \pm 1.77 \mu\text{g/mL}$ (50.25% inhibition). It means bark extract has a stronger capacity as an agent to inhibit tyrosinase. Our finding showed the inhibition from needle extract is much higher than the previous one, analyzed tyrosinase inhibition of ethanol extract of pine needles from three species of pinus sp and showed the inhibitory activity of *Pinus densiflora* was 23%, 25% for *Pinus thunbergii*, and 38% for *Pinus densiflora* [46].

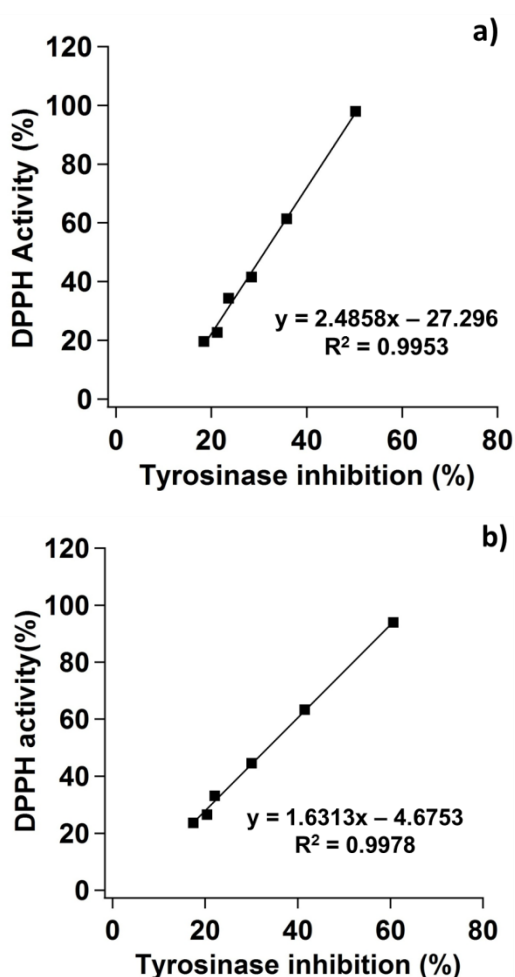


Figure 3. Correlation between antioxidant activity and tyrosinase inhibitory activity of *P. merkusii* needle a) and bark b) extract.

Figure 3 shows high correlation between antioxidant activity and tyrosinase inhibitory activities of needle extract ($R^2 = 0.9953$, $y = 2.4858x - 27.296$) and bark extract ($R^2 = 0.9978$, $y = 1.6313x - 4.6753$), suggesting that antioxidant activity of both extracts play important role in inhibitory activity of tyrosinase enzyme. The antioxidant activity cannot be separated from the secondary metabolite compounds contained in the two extracts. From the preliminary screening results, it can be seen that the needle and bark extracts contained phenolics compounds that have antioxidant bioactivity. In addition, tannins

showed inhibiting tyrosinase enzyme activity [49], [50]. Based on the IC_{50} values, bark extract can be developed as a source of antioxidants or antiaging.

CONCLUSION

In conclusion, this study identified the bioactivity of natural molecules presence in the needle and bark of *P.merkusii*, specifically the antioxidant capacity and tyrosinase inhibitory activity. Although the antioxidant capacity of bark extracts lower than needle extracts, the inhibition tyrosinase activity of barks showed much higher than needle extract. We also found both pine extracts have a higher capacity than several other pine species. In general, needle and bark extracts of *P. merkusii* have the potential to become natural sources of antioxidant and tyrosinase inhibitor for foods and cosmetics.

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