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Research Article

Tyrosinase Inhibition Activity and Phytochemical Screening of *Melaleuca leucadendron* L. Leaves

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Abstract

Melaleuca leucadendron L. is a plant whose almost all parts (bark, leaves, twigs, and fruit) can be used as medicine, such as antioxidants, antifungals, sedative effects, and anti-hyaluronidase. This research was conducted to determine tyrosinase inhibition activity and compound content of *M. leucadendron* leaves. Maceration of *M. leucadendron* leaves was done in methanol, then carried out liquid-liquid fractionation with n-butanol, chloroform, and water. Methanol extract, butanol fraction, chloroform fraction, and water fraction were tested for phytochemical screening and tyrosinase inhibition using L-DOPA substrate with an ELISA plate well reader. The results of the tyrosinase inhibition activity test at concentrations of 100, 1000 and 10000 µg/mL respectively showed that methanol extract 29.532%, 55.227%, 89.583%; butanol fraction 29.313%, 59.174%, 94.737%, chloroform fraction 21.820%, 24.671%; 53.765%; water fraction 24,086%, 47.661%, 91.118%. Inhibition of the tyrosinase enzyme is shown through the IC_{50} value from methanol extract, butanol fraction and water fraction, and kojic acid as a positive control, respectively 645.438 µg/mL, 517.935 µg/mL, 669.403 µg/mL, 50.064 µg/mL. Phytochemical screening showed that the extract and fraction contained tannins, flavonoids, saponins, terpenes, and steroids. These results indicate that the butanol fraction is more potent as an anti-tyrosinase agent than the others.

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INTRODUCTION

The skin is the outermost part of the human body that plays a vital role in body protection. One of the functions of the skin is to protect the body from UV rays[1]. Excessive ultraviolet (UV) light exposure increases the contribution of free radicals known as reactive oxygen species (ROS)[2]. Reactive oxygen species increase pigmentation and cause oxidative stress-induced damage to the melanocytes[3]. Hyperpigmentation disorders are diseases in which patches of skin are darker than the normal surrounding skin, resulting from the upregulated activity of melanin synthesis, increased numbers of melanocytes, and decreased decomposition of melanosomes[4]. Hyperpigmentation conditions can also be caused by certain drugs, hormonal changes, or autoimmune conditions[5].

The enzyme that plays an essential role in the melanin synthesis pathway is tyrosinase. Tyrosinase has the activity of tyrosine hydroxylation, oxidation of L-DOPA (3,4-dihydroxyphenylalanine), and oxidation of hydroxyindole[6]. In the process of melanogenesis, tyrosinase acts as a catalyst in two different reactions, the hydroxylation of tyrosine to L-DOPA and the oxidation of L-DOPA to dopaquinone[7]. Dopaquinone is a highly reactive compound that can spontaneously polarize to form melanin[8].

Bleach acts as an inhibitor of melanin production and is a competitive tyrosinase inhibitor. Various tyrosinase inhibitors are found in cosmetic ingredients to prevent hyperpigmentation, including hyaluronic acid, arbutin, kojic acid, mercury, and

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hydroquinone[9]. This compound has immense whitening power despite having severe side effects such as carcinogenesis, hepatotoxicity, and dermatitis[10]. Hydroquinone not only inhibits tyrosinase activity and destroys melanosomes but also causes necrosis of melanocytes by modifying the membrane structure[11]. This is the potential mechanism of action of hydroquinone as a skin-lightening agent and its toxicity mechanism. The use of this ingredient in cosmetics has been banned since 2001 because of the high risk of carcinogenesis in case of prolonged exposure to hydroquinone[12].

Several researchers prefer to identify inhibitors from natural sources due to their less toxicity and better bioavailability, especially for food, cosmetic and medicinal applications[13]. The class of flavonoid compounds that have tyrosinase inhibitory activity is quercetin from the flavonol group. Flavonoid compounds have tyrosinase inhibitor and chelating activity Cu, where the hydroxyl groups on the A and rings B inhibit the action of tyrosinase[14]. Besides flavonoids, other polyphenols, also known as tyrosinase inhibitors, include coumarin, stilbenes derivatives[15], terpenoid derivatives[16], and lignans[17].

In previous studies, the antioxidant activity test of *Melaleuca leucadendron* L. leaves was extracted with methanol and then fractionated with chloroform and butanol. The total phenolic that has been carried out in previous studies was 289.23 ± 5.21 µg GAE/g in methanol extract, 107.36 ± 1.88 µg GAE/g in chloroform extract, and 508.43 ± 2.33 µg GAE/g in butanol extract. While the IC_{50}- obtained in the *M. leucadendron* extract as an antioxidant was 14.5 µg/mL in methanol extract, 50.3 µg/mL in chloroform extract, and 10.1 µg/mL in butanol extract[18]. Based on previous studies, the tyrosinase inhibition test on *M. leucadendron* leaves has never been carried out. This test is necessary because a high total phenolic and a low IC_{50} value of antioxidants can act as anti-tyrosinase. We hope this research can contribute to developing new safe, efficient anti-tyrosinase agents to prevent hyperpigmentation disorders.

MATERIALS AND METHODS

Materials

Melaleuca leucadendron dry leaves were collected from Balai Penelitian Tanaman Rempah dan Obat (Balitro), which has been identified at the Botanical Garden Plant Conservation Center, Lembaga Ilmu Pengetahuan Indonesia (LIPI), number of the certificate B-1222/IPH3/KS/X/2020 (**Figure 1**). Chemical reagents such as methanol 75%, L-DOPA (Sigma), tyrosinase (Sigma), kojic acid (Sigma), chloroform, butanol, distilled water, HCl 2 N, Dragendorff reagent, Mayer reagent, Wagner reagent, Bouchardat reagent, HCl, $NaNO_2$ 5%, $AlCl_3$ 10%, NaOH 1 N, $FeCl_3$ 1%, NaOH 2 N, ether, H_2SO_4, potassium dihydrogen phosphate, dimethyl sulfoxide (DMSO) (Merck), and phosphate buffer (pH 6.5). At the same time, the equipment used includes a digital analytical scale, rotary vacuum evaporator, multi-well plate reader (ELISA), multilevel fractionation device, pH meter, and incubator.

a **b**

Figure 1. *Melaleuca leucadendron* leaves (**a**) simplicia powder of *M. leucadendron* leaves (**b**).

Methods

Extraction and fractination

Melaleuca leucadendron dry leaves were ground to obtain 500 g of sample powder for extraction. Methanol 75% (5 L) was used as the solvent in the maceration extraction of the samples for 3 x 24 hours. The crude methanol extracts were then dried using a rotary evaporator. Liquid-liquid fractionation was conducted using distilled water, butanol, and chloroform to the methanol extract to obtain fractions with different polarities, then dried using a rotary evaporator.

Phytochemical screening

Extracts and three fractions were carried out in a phytochemical screening test to identify alkaloids, flavonoids, tannins, saponins, steroids/triterpenoids using the classical method[19].

Tyrosinase inhibitory assay

Tyrosinase inhibitory activity was evaluated based on inhibition of the sample (diluted in DMSO) to diphenolase activity. The assay was carried out using an ELISA plate well reader with tyrosinase enzyme, L-DOPA as the substrates, phosphate buffer pH 6.5, with three repetitions[20]. Kojic acid was used as a positive control. The following **Equation 1** can calculate the percentage of tyrosinase inhibitory activity:

$$\text{Inhibition (\%)} = [1 - \frac{(A-C)}{(B-D)}] \text{ x } 100\% \quad \ldots [1]$$

In which,

A: Absorbance of the sample

B: Absorbance of blank

C: Absorbance of sampel control

D: Absorbance of blank control

The IC_{50} value can be calculated using a linear regression equation, sample concentration (x-axis), and %-inhibition (y-axis). From the equation $y = a \ln (x) + b$, the IC_{50} value can be calculated using the **Equation 2**.

$$\ln IC_{50} = \frac{50-b}{a} \quad \ldots [2]$$

RESULTS AND DISCUSSION

The yields of the extracts and their respective fractions are presented in **Table I**. The extraction method is maceration because the equipment used is simple and easy. Methanol 75% was used as a solvent because it can attract the highest phenolic compounds and has a low antioxidant IC_{50} value[18]. During maceration, stirring is carried out so that the pollen liquid penetrates the cell wall and enters the cell cavity containing the active substance. The difference in concentration between the solution inside and outside the cell causes a more concentrated solution to be pushed out so that the metabolite compound can be extracted entirely[21].

Based on the results of phytochemical screening obtained on *M. leucadendron* leaves extract containing flavonoids, saponins, tannins, and steroids/triterpenoids and negative results in the alkaloid test (**Table II**). Previous research[22] showed that *M. leucadendron's* methanol fraction contains alkaloid compounds, flavonoids, saponins, tannins, steroids, and triterpenoids. The difference in results obtained is due to the use of hexane solvent when maceration. The water fraction shows negative results in the steroid/triterpenoid test; this is because terpenoids can be extracted using non-polar solvents (ether, hexane, chloroform), while in the form of glycosides (generally from triterpenes) the solubility is more remarkable in polar solvents (ethanol, methanol).

Table I. The yield of the extract and fraction of *M. leucadendron* leaves

Extracts/Fraction	Powder weight (g)	Extract weight (g)	Yield (%)
Methanol	500	82.02	16.04
Chloroform	40	12.6	31.5
Water	40	21	52.5
Butanol	40	6.5	16.25

Table II. Phytochemical screening test results of *M. leucadendron* leaves

Test	Sample			
	ME	BF	CF	WF
Saponin	+	+	+	+
Alkaloid				
Mayer	-	-	-	-
Wagner	-	-	-	-
Dragendorff	-	-	-	-
Tannins	+	+	+	+
Flavonoids	+	+	+	+
Steroids-terpenoids	+	+	+	-

ME: methanol extract; BF: butanol fraction; CF: chloroform fraction; WF: water fraction

The activity of tyrosinase inhibitors is one of the parameters of skin lightening agents. The mechanism of tyrosinase inhibitors is to decrease skin pigmentation by inhibiting the catalytic of the enzyme to the pigmentation associated with melanin production in the melanogenesis pathway[23]. Kojic acid is used as a positive control because it is one of the tyrosinase inhibitors used as a cosmetic ingredient. It is a skin protector from the ultraviolet sun and can whiten the skin. Kojic acid prevents the formation of melanin in human melanocytes due to the reversible inhibition of tyrosinase, but it has some side effects, such as skin irritability and instability[24].

The butanol fraction had the highest tyrosinase inhibition value (94.737±0.767%) at 1000 µg/mL concentration. However, kojic acid as a positive control show better inhibition at a concentration of 500 µg/mL with tyrosinase inhibition value of 91.155±0.228%. Measurement of the IC_{50} value was carried out on methanol extract, butanol fraction, water fraction, and kojic acid, while the chloroform fraction was not carried out because of the low percentage value of inhibition at a concentration of 1000 µg/mL (**Table III**).

Table III. Tyrosinase inhibitory of *M. leucadendron* leaves

Sample	Concentrations (µg/mL)	Tyrosinase Inhibition (%)
Methanol extract	100	29.532 ± 0.713
	1000	55.227 ± 1.081
	10000	89.583 ± 0.110
Butanol fraction	100	29.313 ± 0.920
	1000	59.174 ± 1.299
	10000	94.737 ± 0.767
Chloroform fraction	100	21.820 ± 1.245
	1000	24.671 ± 1.245
	10000	53.765 ± 0.444
Water fraction	100	24.086 ± 1.271
	1000	47.661 ± 0.228
	10000	91.118 ± 0
Kojic acid	500	91.155 ± 0.228

Values are expressed as mean ± SD of triplicate measurements

The IC_{50} values of tyrosinase inhibition of all samples are presented in **Table IV**. Butanol fraction had the lowest IC_{50} value with 517.94 µg/mL. Nevertheless, kojic acid as a positive control was more potent with IC_{50} 50.06 µg/mL. The butanol fraction is more active than other extracts and fractions, while in previous research[18], the total phenolic content was reported to be more significant, 508.43±2.33 µg GAE/g extract, and the antioxidant IC_{50} value of 4.8 µg/mL. From the research results, it can be seen that there is a correlation between total phenolic and antioxidant activity with tyrosinase activity.

Table IV. IC_{50} of tyrosinase inhibitory of *M. leucadendron* leaves

Sample	Concentrations (µg/mL)	Tyrosinase inhibition (%)	IC_{50} (µg/mL)
Methanol extract	500	47.11±0.39	645.44
	1000	54.18±0.51	
	1500	58.38±0.53	
	2000	58.18±0.84	
	2500	59.71±0.75	
Butanol fraction	500	48.85±0.57	517.94
	1000	60.83±0.84	
	1500	69.65±0.71	
	2000	73.18±0.29	
	2500	74.37±1.03	
Water fraction	500	48.23±0.25	669.40
	1000	51.66±0.53	
	1500	59.91±0.58	
	2000	66.63±0.85	
	2500	67.99±0.54	
Kojic acid	31.25	33.23±0.41	50.06
	62.5	51.74±0.84	
	125	79.20±0.08	
	250	89.65±0.14	
	500	94.84±0.05	

Values are expressed as mean ± SD of triplicate measurements

CONCLUSION

Inhibition of the tyrosinase enzyme is shown through the IC_{50} value from methanol extract, butanol fraction, and water fraction was 645.44 µg/mL, 517.94 µg/mL, 669.40 µg/mL, respectively. As a positive control, the IC_{50} value of kojic acid was 50.06 µg/mL. Phytochemical screening showed that the extract and fraction of *M. leucadendron* leaves contained tannins, flavonoids, saponins, terpenes, and steroids. These results indicate that the butanol fraction of *M. leucadendron* leaves is the most potent anti-tyrosinase agent compared to the others.

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AUTHORS' CONTRIBUTION

Munawarohthus Sholikha: conceptualization, methodology, get a grant for funding, supervised the experimental works, writing and review. **Ainun Wulandari**: contributed in the experimental works and assisted for manuscript writing.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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