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Sun protection factor and tyrosinase inhibitory activity of several plant secondary metabolites

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ABSTRACT

Severe exposure to ultraviolet (UV) rays leads to skin damage, including hyperpigmentation, freckles, melanoma, age spots, and melasma, all of which are related to the skin pigment enzyme, tyrosinase. Prevention can be achieved by avoiding harsh UV rays and inhibiting tyrosinase catalytic activity. Many compounds have been developed for the treatment of such conditions; however, most come with unwanted side effects. The purpose of this study was to determine the sun protection factor (SPF) value and tyrosinase enzyme inhibitory activity of plant secondary metabolites with high antioxidant activity, namely rutin, catechin, niazirin, piperine, quercetin, and quercitrin, as potential alternatives. Both tests were carried out using UV-Vis spectrophotometry. SPF determination was performed by observing the absorbances across a wavelength range of 290 to 320 nm. The determination of anti-tyrosinase activity was conducted by measuring dopachrome at 490 nm after enzymatic reactions and calculating the IC50 value. In the SPF assay, benzophenone-3 was used as the standard, and piperine, rutin, quercetin, and quercitrin exhibited high protective abilities with SPF values above 30 at 500 µg/mL. In the tyrosinase inhibition assay, kojic acid as the standard showed a strong potential for inhibition with an IC_{50} of 33.65 µg/mL, while quercetin, rutin, and piperine exhibited weaker inhibitory potential with IC50 values of 178.44, 271.73, and 347.62 µg/mL, respectively. On the other hand, quercitrin and niazirin showed little to no tyrosinase inhibition activity. However, catechin demonstrated more catalytic activity towards the enzyme. In conclusion, quercetin, rutin, and piperine have the potential to be developed as active ingredients to protect the skin from UV-induced damage due to their satisfactory SPF values and tyrosinase inhibition activity.

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1. Introduction

Indonesia, being a tropical country, is continuously exposed to solar radiation throughout the day. The Ultraviolet (UV) index, measured by the Indonesian Meteorological, Climatological, and Geophysical Agency (abbreviated BMKG), indicates the level of UV radiation exposure related to human health in the country. Often, Indonesia experiences a high UV index ranging from 8 to 11, classified as "very high," and in some areas, it may even reach above 11, categorized as "extreme." Prolonged exposure to such high UV levels can lead to skin damage, including hyperpigmentation and an increased risk of skin cancer, such as melanoma (Permana et al., 2022). To prevent these skin damages, two methods can be employed. The first involves avoiding harsh UV radiation exposure by using sunscreen with a sufficient sun protection factor (SPF). The second approach is to directly inhibit the catalytic activity of the enzyme tyrosinase, which is involved in melanin synthesis (Pillaiyar et al., 2017).

One effective method to reduce UV radiation damage is the use of sunscreen, which contains ingredients that protect the skin by reflecting and absorbing UV rays (Permana et al., 2022). However, many commercial sunscreen products utilize synthetic chemicals as their main components, which can lead to unwanted side effects with prolonged use. Consequently, there is a need to develop safer formulations using natural ingredients (Yani and Dirmansyah, 2021). Commonly used sunscreen ingredients, such as oxybenzone, octinoxate, and benzophenone-3, exhibit high UV absorption but are allergenic and can disrupt hormonal balance in humans. Oxybenzone, in particular, affects estrogen, progesterone, and androgen receptors and is a common photoallergen among UV filters. Meanwhile, octinoxate is associated with mild photoallergic properties and similar endocrine effects to oxybenzone. Benzophenone-3 was named the contact allergen of the year in 2014. Therefore, the development of alternative ingredients should carefully consider both their benefits and risks (Siller et al., 2018).

Another approach involves using tyrosinase inhibitors, which have been extensively studied. Hydroquinone, arbutin, kojic acid, L-ascorbic acid, and ellagic acid are examples of commonly used skin whitening agents with tyrosinase inhibitory properties. However, these compounds have drawbacks and potential side effects. Hydroquinone, for instance, may cause mutations in mammalian cells and lead to irritation, contact dermatitis, leukoderma, and ochronotic effects. Arbutin's natural form is chemically unstable and can release hydroquinone, which may pose a toxicity risk to the bone marrow. The use of kojic acid is limited due to its instability during storage and potential carcinogenicity. L-ascorbic acid is easily degraded and heat-sensitive, while ellagic acid has poor bioavailability due to its insolubility (Pillaiyar et al., 2017). Consequently, there is a significant need for the development of new and effective anti-tyrosinase agents.

In this study, the researchers investigated six plant secondary metabolites, namely rutin, catechin, niazirin, piperine, quercetin, and quercitrin, for their sun protection factor (SPF) and tyrosinase inhibitory activities. Both activities were assayed using UV-Vis spectrophotometry. SPF determination involved observing absorbances within the wavelength range of 290 to 320 nm, while the anti-tyrosinase activity was assessed by measuring dopachrome at 490 nm after enzymatic reactions and calculating the IC_{50} value.

2. Materials and methods

2.1. Materials

Purified fraction of Rutin CAS No. 153-18-4, Niazirin CAS No. 122001-32-5, Catechin CAS No. 88191-48-4, Piperine CAS No. 94-62-2, Quercetin CAS No. 117-39-5, and Quercitrin CAS No. 522-12-3 obtained from MarkHerb. Benzophenone-3 CAS No. 5270-74-6, Kojic Acid CAS 501-30-4, L-3,4-Dihydroxyphenylalanine methyl ester hydrochloride (L-DOPA) CAS No. 1421-65-4, Tyrosinase from mushroom CAS No. 9002-10-2, and NaOH CAS No. 1310-73-2 purchased from Sigma-Aldrich. Dimethyl sulfoxide CAS No. 67-68-5, Natrium dihydrogen phosphate-Monohydrate CAS No. 10049-21-5, and di-Sodium hydrogen phosphate CAS No. 7558-79-4 from Merck.

2.2. Sun protection factor assay

2.2.1. Preparation of reference standard solution

For the SPF assay, a benzophenone-3 standard was employed. Initially, 10 mg of benzophenone-3 was dissolved in 100 µL of DMSO to create a stock solution with a concentration of 100,000 µg/mL. From this stock, smaller quantities were prepared at 50, 200, and 500 µg/mL by diluting 0.5, 2, and 5 µL of the stock solution, respectively, with DMSO until the final volume reached 1 mL.

2.2.2. Preparation of sample solution

Stock solutions of 100,000 µg/mL were prepared from 10 mg of rutin, niazirin, catechin, piperine, quercetin, and quercitrin. From the stock solution, three dilutions with concentrations of 50, 200, and 500 µg/mL were made using DMSO to a volume of 1 mL for each.

2.2.3. Analysis of SPF using UV-Vis spectrophotometer

The assay was conducted using a 96-well microplate, with each test performed in triplicate. In each well, 200 µL of the sample, standard, or blank was added. The blank wells contained only DMSO, while the other wells were filled with their respective solutions. The microplate was then placed into the UV-Vis Spectrophotometer, and the absorbances were measured at 5 nm wavelength intervals within the range of 290 to 320 nm (Khan, 2018). To calculate the SPF value, a normalized function combining the erythemogenic effect and intensity of solar constants by Sayre et al. (1979) was used, along with the Mansur equation.

$$SPF = CF \times \sum_{320}^{290} EE_{(\lambda)} \times I_{(\lambda)} \times Abs_{(\lambda)}$$

where,
CF = Correction Factor (=10)
EE = Erythemogenic Effect
I = Intensity of Solar Constant
Abs = Absorbance

2.3. Tyrosinase inhibition assay

The method for this assay was adapted from Wijaya et al. (2018) with some modifications.

2.3.1. Preparation of phosphate buffer pH 6.8 solution

The buffer solution was prepared by mixing 127 mg of 0.0179 M Na_2HPO_4 and 221.5 mg of 0.0321 M NaH_2PO_4. Both compounds were dissolved in distilled water until the final volume reached 50 mL. The pH of the solution was adjusted by carefully adding NaOH drop by drop while periodically checking the pH value until it reached pH 6.8. Additional phosphate buffer solutions were also prepared to ensure an adequate supply of materials

2.3.2. Preparation of DMSO 5%

A 50 mL solution of DMSO 5% was prepared by combining 2.5 mL of DMSO with 47.5 mL of phosphate buffer at pH 6.8. The mixture was thoroughly mixed using a vortex to ensure homogeneity.

2.3.3. Preparation of L-DOPA solution

The required L-DOPA solution concentration was 2.5 mM. Considering a dilution factor of five, the final concentration prepared was 12.5 mM. To achieve this, 2.5 mg of L-DOPA was dissolved in 1 mL of phosphate buffer at pH 6.8. Additional L-DOPA solutions were prepared to ensure an adequate supply of material.

2.3.4. Preparation of tyrosinase enzyme

The tyrosinase enzyme was prepared under the required conditions. To ensure protection from light, all tubes were wrapped with aluminium foil before the preparation process, and ice blocks were prepared to maintain the enzyme's stability. The desired concentration of tyrosinase enzyme was 100 U/mL, which was achieved by diluting it from a 25,000 U/mL stock solution. Due to the dilution factor, the final concentration reached 500 U/mL. The mixture was prepared by diluting 20 L of the stock solution with the phosphate buffer at pH 6.8 to make a 1 mL solution. Additional tyrosinase enzyme solutions were prepared to avoid any shortage of material.

2.3.5. Preparation of standard solution

From a kojic acid stock solution with a concentration of 0.5 M, a series of dilutions was prepared to achieve concentrations of 10, 25, and 50 g/mL. These dilutions were made in five times their concentration due to the dilution factor, resulting in final concentrations of 50, 125, and 250 g/mL. For each concentration, 0.7, 1.7, and 3.4 L of the stock solution were used, respectively. Each of these amounts was then diluted with phosphate buffer at pH 6.8 to make a 1 mL solution for each concentration.

2.3.6. Preparation of sample solution

The stock prepared from the SPF method was used to create a series of dilutions with concentrations of 50, 200, and 500 g/mL. Due to the dilution factor, the concentrations of the samples were multiplied by five, resulting in final concentrations of 250, 1000, and 2500 g/mL. For each concentration, it required 0.5, 10, and 25 L of the stock solution, respectively. In the case of piperine, 1, 20, and 50 L were taken from the stock solution to obtain each respective concentration. Afterward, all the solutions were diluted with DMSO 5% to reach a final volume of 1 mL for each sample

2.3.7. Tyrosinase inhibitory activity measurement

In the tyrosinase inhibition assay, four variables were measured: A) Blank with tyrosinase enzyme, B) Blank without tyrosinase enzyme, C) Sample with tyrosinase enzyme, D) Sample without tyrosinase enzyme. Each well was filled with a total of 200 L of solution, as shown in Table 1.

The assay was performed in triplicate for both the samples or standards and in duplicate for the wells without samples or standards. Each well was filled with its corresponding materials, and then the plate was incubated at 37°C for 10 minutes. After incubation, the absorbance of each well was measured at a wavelength of 490 nm (Wijaya et al., 2018). The tyrosinase inhibition percentage was calculated using the following formula.

$$Tyrosinase\ inhibition\ (\%) = \left(\frac{(Abs_A - Abs_B) - (Abs_C - Abs_D)}{Abs_A - Abs_B}\right) \times 100$$

where,

Abs_A = Absorbance of blank with tyrosinase
Abs_B = Absorbance of blank without tyrosinase
Abs_C = Absorbance of sample or standard with tyrosinase
Abs_D = Absorbance of sample or standard without tyrosinase

Linear regression for each sample was performed by plotting the concentration on the x-axis and tyrosinase inhibition percentage on the y-axis. The median inhibitory concentration or IC_{50} was calculated by determining the linear regression equation for each sample. The values of 'a' and 'b' within the formula were obtained from the aforementioned equation.

$$y = ax + b$$

$$IC_{50} = \frac{(50 - b)}{a}$$

Table 1. Well composition for tyrosinase inhibition assay

Material	Volume (L)			
	A	B	C	D
Phosphate buffer pH 6.8	120	160	80	120
Sample or Standard Solution	-	-	40	40
Tyrosinase Enzyme	40	-	40	-
L-DOPA	40	40	40	40

2.4. Statistical analysis

Data from the sun protection factor and tyrosinase inhibition assays were expressed as Mean±SD. Kruskal-Wallis one-way analysis of variance followed by the Dunn post-hoc test was performed to assess significant differences between groups. A result was considered significant when $p<0.05$. The statistical analysis was conducted using R Studio.

3. Results and discussion

3.1. Sun protection factor activity

The metabolites samples can be ranked based on their SPF values, with higher SPF values indicating more effective protection against destructive skin damage caused by ultraviolet radiation (UVR). Additionally, higher concentrations of these compounds may result in stronger protection capabilities (Tahir et al., 2021). The classification is based on the study published by Lionetti and Rigano in 2017.

The results from the SPF assay are shown in Table 2. The standard benzophenone-3 exhibited high protection ability at 200 and 500 g/mL, with SPF values of 31.84 0.17 and 31.67 0.07, respectively. At 50 g/mL, only piperine showed UVR protection with a lower ability at an SPF value of 10.71 0.88. At 200 g/mL, rutin displayed low protection with an SPF value of 13.95 0.14. On the other hand, quercitrin and quercetin provided medium protection with SPF values of 18.76 0.12 and 20.69 0.06, respectively. At the same concentration of 200 g/mL, only piperine demonstrated high protection ability with an SPF value of 32.93 0.11. At 500 g/mL, rutin, piperine, quercetin, and quercitrin all exhibited high protection with SPF values above 30, specifically 30.76 0.33, 32.67 0.07, 33.50 0.18, and 33.01 0.12, respectively. However, both niazirin and catechin showed low to no SPF values across all three concentrations, suggesting a lack of protection against UVR.

Table 2. SPF values calculated at 50, 200, and 500 g/mL

Sample	SPF at 50 µg/mL		SPF at 200 µg/mL		SPF at 500 µg/mL	
Benzophenon-3 (standard)	5.87	0.31	31.84	0.17	31.67	0.07
Rutin	1.99	0.15	13.95	0.14	30.76	0.33
Niazirin	0.19	0.12	0.19	0.10	0.11	0.09
Catechin	0.68	0.11	0.70	0.12	2.04	0.07
Piperine	10.71	0.88	32.93	0.11	32.67	0.07
Quercetin	3.50	0.23	20.69	0.06	33.50	0.18
Quercitrin	2.84	0.07	18.76	0.12	33.01	0.12

Values are expressed as mean standard deviation of triplicate measurements

Based on the statistical analysis, SPF values of rutin, piperine, quercetin, and quercitrin are not significantly different from that of benzophenone-3. These four compounds show comparable UVR absorbance to the positive control, making them excellent candidates for sunscreen ingredients. However, further research is necessary to evaluate their safety and effectiveness *in vivo*. It is important to consider factors such as SPF value reduction due to dilution, stability loss, formulation amount used, individual skin characteristics, and the area of application. Therefore, combining these ingredients with other materials may provide optimal protection.

The correlation between the plant secondary metabolites and their UV absorbances is based on their molecular structures. Compounds that contain chromophores with conjugated double bonds have the ability to absorb UV light, thereby preventing more radiation from passing through. Among the tested flavonoids, rutin, quercetin, and quercitrin showed high SPF values. Flavonoids, in general, have photoprotective potential due to the presence of conjugated double bonds in their structures (Jos et al., 2016).

Rutin contains a disaccharide, rutinose, attached to carbon 3 in the flavonolic aglycone (Ganeshpurkar and Saluja, 2017).

Quercetin and quercitrin are structurally similar to rutin, with quercetin having a hydroxyl group and quercitrin having a rhamnoside in position carbon 3 (Materska, 2008). The chromophores present in these structures are carbonyl, *s-trans* enone, and benzene, with some hydroxyl groups acting as auxochromes. Studies have shown that rutin and quercetin have SPF activity in UVB and protection against UVA, with quercetin exhibiting additional absorption in the UVA region. These two compounds have also been observed to synergize with titanium dioxide, increasing the SPF value and are considered photostable (Jos et al., 2016).

Furthermore, research by Chopra et al. (2017) indicates a significant correlation between the amount of flavonoids and phenolic contents with SPF. Hence, a higher content of flavonoids in a product is likely to result in a higher SPF value. Piperine consists of a piperidine moiety attached to a carbonyl amide linkage, side chains with conjugated double bonds, and a methylenedioxyphenyl (MDP) ring. Some chromophores in piperine's structure include the carbonyl between piperidine and side chain, the C=C bonds throughout the side chain, an s-*cis* diene connecting the side chain to the MDP ring, and a benzene in the MDP ring. On the other hand, catechin is a polyphenol, more specifically a flavan-3-ols, and is composed of a C3 hydroxyl group and two benzenes connected to a dihydropyran heterocyclic ring. The chromophores present in catechin's structure are the two benzene rings and an alkene. As for niazirin, its structure consists of glycosyl and cyanomethyl moieties attached to a phenolic structure. The only chromophores present in catechin and niazirin are an alkene and benzenes, which exhibit little to no UV absorption, mostly absorbing in the wavelength of 190 nm and 210 nm, respectively (Berova et al., 2007)

3.2. Tyrosinase inhibition assay

Tyrosinase inhibition activity can be assessed by quantifying the IC_{50} or median inhibitory concentration. The IC_{50} represents the concentration required to inhibit 50% of the tyrosinase enzyme. Based on a study by Tahir et al. (2021), the IC_{50} values can be categorized as follows:

- IC_{50} < 100 g/mL: Strong potential for tyrosinase inhibition
- IC_{50} 100-450 g/mL: Weak potential for tyrosinase inhibition
- IC_{50} 450-700 g/mL: Very weak potential for tyrosinase inhibition
- Smaller IC_{50} values indicate greater tyrosinase inhibitory activity

In this study, the tyrosinase inhibition activity of rutin, niazirin, catechin, piperine, quercetin, and quercitrin were measured using both %TI (tyrosinase inhibition percentage) and IC_{50} values. The resulting data for both measurements can be found in Table 3.

The test results of plant secondary metabolites were compared with the positive control, kojic acid, which had the lowest IC_{50} value of 33.65 g/mL, indicating strong potential for tyrosinase inhibition. Among the tested compounds, quercetin, rutin, and piperine showed weak potential, with IC_{50} values of 178.44 µg/mL, 271.73 µg/mL, and 347.62 µg/mL, respectively. On the other hand, niazirin and quercitrin exhibited very weak to no inhibitory potential with their IC_{50} values being higher than 500 g/mL. The statistical analysis showed no significant difference between the IC_{50} values of kojic acid and rutin, piperine, and quercetin. This suggests that those three compounds have similar tyrosinase inhibition potential with the standard.

Rutin, quercetin, and quercitrin are believed to possess tyrosinase inhibition activity due to the presence of phenolic and hydroxyl groups in their structures. These compounds can compete with L-DOPA in the enzyme, tightly binding to copper ions and amino acid residues near the active site of tyrosinase (Si et al., 2012). The presence of a carbonyl group at C4 of ring C, a hydroxyl group at C3 of ring C, a hydroxyl group at C3 of ring B, and a hydroxyl group at C7 of ring A are also considered essential for competitive inhibition, as they chelate copper ions in tyrosinase (Ş hretoğlu et al., 2018). Piperine, with a carbonyl group in its structure, is also expected to exhibit tyrosinase inhibition activity.

Table 3. %TI and IC_{50} of samples from tyrosinase inhibition assay

Sample	Concentration (µg/mL)	%TI	IC_{50} (µg/mL)
Kojic Acid (standard)	10	3.10 0.18	33.65
	25	24.59 4.79	
	50	87.82 1.51	
Rutin	50	27.48 2.78	271.73
	200	55.80 1.88	
	500	62.21 0.99	
Niazirin	50	27.39 2.35	>500
	200	28.7 1.73	
	500	32.35 1.90	
Catechin	50	25.07 2.03	>500
	200	27.24 2.72	
	500	-35.36 5.96	
Piperine	50	24.75 2.30	347.62
	200	51.80 5.85	
	500	55.66 1.71	
Quercetin	50	29.98 1.04	178.44
	200	61.75 1.34	
	500	81.21 0.39	
Quercitrin	50	9.43 5.21	>500
	200	25.09 1.79	
	500	31.45 7.16	

Values are expressed as mean standard deviation of triplicate measurements

However, the assay using 500 g/mL catechin showed an increase in absorbance, indicating higher absorbance than the blank group. Catechin is also shown to be significantly different with kojic acid. Molecular docking tests revealed that catechin can act like a substrate, compete with L-DOPA, and activate tyrosinase activity. It binds to tyrosinase active sites through π-bonds, van der Waals force, and hydrogen bonds. Compared to L-DOPA, catechin formed more π-bonds with residues of amino acids near the tyrosinase active site, indicating stronger binding to tyrosinase. As a result, instead of inhibiting tyrosinase activity, catechin catalyzes the activity even further (Ma et al., 2022).

In summary, none of the tested plant secondary metabolites could match the tyrosinase inhibition of the standard, kojic acid.. However, there are promising inhibitory values observed from rutin, piperine, and quercetin. Further research and investigations are needed to fully understand their potential as tyrosinase inhibitors.

4. Conclusion

In this study, the sun protection factor and tyrosinase inhibition properties of six compounds derived from plants were examined. Quercetin, piperine, and rutin demonstrated SPF and

tyrosinase inhibition activities comparable to the standard, indicating their potential candidacy as active ingredients for reducing UV-induced skin damage. Quercitrin showed satisfactory results in the SPF assay, whereas niazirin exhibited little to no SPF values and displayed no tyrosinase inhibition activity. Catechin, on the other hand, was observed to be an activator of the tyrosinase enzyme, catalyzing the reaction further. Overall, quercetin, piperine, and rutin hold promise as effective compounds for skin protection, but further research is needed to explore their full potential and safety in sunscreen formulations.

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Conflict of interest

The authors declare there is no conflict of interest in this study.

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