Tyrosinase Inhibitory and Sunscreen Activity of *Averrhoa bilimbi* Leaves N-Hexane Fraction

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Abstract. Tyrosinase is a multi-copper enzyme found in a variety of species that plays an important role in melanogenesis and enzymatic browning. As a result, the epidermis of the body requires a substance that inhibits the activity of the enzyme tyrosinase. Polyphenols and flavonoids in Averrhoa bilimbi, also known as Belimbing Wuluh in Indonesia, block tyrosinase enzymes. The purpose of this study is to investigate total phenolic content (TPC), total flavonoid content (TFC), tyrosinase inhibitors, and Sun Protecting Factor (SPF) activity in the N-hexane fraction of Averrhoa bilimbi leaves. TPC using a Folin–Ciocalteu (TFC) reagent, Tyrosinase inhibitor activity by mushrooms tyrosinase test, and evaluation in the range 290-320 nm for SPF, 290-315 nm for transmitted erythema, and 320-370 nm for transmitting hyperpigmentation were all used in this study. The study found 68.26 8.58 mg GAE/g sample in TPC of the N-hexane fractions. The n-hexane fractions had a TFC of 112.31 7.22 mg RE/g material. The SPF of 100, 200, and 300 ppm N-hexane fraction concentrations is 5.66, 11.69, and 17.58, respectively. The transmitted erythema has a concentration of 100, 200, and 300 parts per million (ppm), or 130.69 percent, 32.49 percent, and 8.92 percent, respectively. Transmission pigmentation is 17.99 percent, 6.90 percent, and 2.75 percent for 100, 200, and 300 ppm. It was derived from the sunscreen type's sunblock rating. Tyrosinase inhibition had an Ic50 of 470.92 82.31 g/mL, which was higher than kojic acid's 16.68 g/mL. The n-hexane fractions' flavonoids and polyphenol compounds operate as tyrosinase inhibitors and sunscreens.

INTRODUCTION

Chronic skin exposure to ultraviolet (UV) light may cause aging, hypopigmentation, erythema, burns, hyperpigmentation, cracks, immunological destruction, dermatitis, wrinkles, and the most severe significant skin cancers [1]. Natural chemicals such as polyphenol as flavonoids are more effective than synthetic chemicals due to lasting beneficial effects, especially against damage to the skin produced by free radicals and UV-blocking. Skin requires several means of protection to have continuing benefits and avoid chronic conditions like cancer.

Tropical plants are associated with many medicinal properties: Averrhoa bilimbi, a native cultivar known as *Averrhoa bilimbi. Averrhoa bilimbi* is a natural ingredient commonly used as traditional herbal medicine for skin health. The total phenolic content of Averrhoa bilimbi fruits was investigated using a methanolic extract [2]. Total phenolic content was shown to correlate with antioxidant activity [3] strongly. Flavonoids are phenolic compounds with potent antioxidant effects [3]. There are no publications on the action of the N-hexane fraction from Averrhoa bilimbi on total flavonoid content (TFC), total phenolic content (TPC), sun protection factor (SPF), and tyrosinase inhibitory activity.

The primary goal of this study is to use the Folin-Ciocalteu reagent and a colorimetric test to evaluate total phenolic and total flavonoid content and UV spectrophotometry techniques to estimate the SPF of sunscreens [4][5]. The activity of tyrosinase inhibitors was determined using a mushroom tyrosinase test. The sunscreen preparation has been produced because it was discovered that UV radiation produces considerable damages to the skin, for instance, sunburn, melanoma, unnaturally wrinkling, pigmentation of the skin, and coarsening of the skin surface. It is vital to

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apply sunscreen preparations. These treatments contain chemicals that have the action to inhibit UV radiation from accessing the skin. Three sunscreens are categorized according to their instrument, i.e., ultraviolet reflector, ultraviolet absorbent, and tanning compounds. Tanning chemicals are substances that form a brown complex combined with the keratin of the corneal layer.

Furthermore, whitening procedures remain essential to decrease peculiarly pigmented skin. They impede the new formation of melanin by blocking the oxidative polymerase enzyme. As is widely known, melanin is a dark skin pigment. The Averrhoa bilimbi extracts may comprise a chemical that can prevent oxidative polymerization from lessening the melanin synthesis. According to the previous description, Averrhoa bilimbi leaves may contain various compounds that absorb UV light, generate a brown complex with keratin, and prevent melanin synthesis. Thus, Averrhoa bilimbi leaves can be utilized for raw resources in sunscreen and skin whitening processes. The investigation on "The research of whitening and sun screening compounds in Averrhoa bilimbi leaves" was done to prove the theory.

METHOD

Material

At the Stifar Semarang laboratory of pharmaceutical biology, a botanist identified fresh Averrhoa bilimbi leaves. Methanol, Ethanol, Folin-Ciocalteu, aluminum chloride, Natrium Carbonate, Natrium Acetate, Rutin, Gallic acid, Kojic acid, tyrosinase enzyme from mushroom, L-tyrosine as a substrate–Sigma–Aldrich (St. Louis, MO, USA) (St. Louis, MO, USA). Instruments utilized in the experiment included a UV-Vis spectrophotometer (Shimadzu UV-1280, Japan) and a UV-Vis spectrophotometer (Shimadzu UV-1280, Japan) (Shimadzu UV-1280, Japan).

Sample preparation

The Averrhoa bilimbi leaves used are mature leaves, have an even green color, and then are extracted. Extraction and fractionation of Averrhoa bilimbi3 kilograms Averrhoa bilimbi leaves were dried. For 24 hours, 300 g of powder macerated in 96% ethanol, residues were macerated three times using the same solvent. The extract was accumulated and evaporated at 60°C and 100 RPM till a thick extract was obtained. The viscous extract was fractionated with a nonpolar solvent to a polar solvent. N-hexane, ethyl acetate, and water were utilized as solvents. The nonpolar N-hexane faction was employed as the study's sample.

Determination of Total Phenolic Content (TPC)

Determination of TPC modifications has been introduced to the phenolic content determination of earlier material. [6] validated by [7]. With 0.5 mL sample and 0.04 mL Folin-Ciocalteureagent, incubate for 4-8 minutes. 4.0 mL of 7% Natrium Carbonate was also added, mixed with distilled water, and incubated for two hours at room temperature. At 750 nm, a UV-Vis spectrophotometer was used to take measurements. A gallic acid standard (mg GAE/g) was used to create a calibration curve.

Determination of Total Flavonoid Content (TFC)

The Chang method with modification [8] was used to calculate TFC, validated by [9] with an alteration. 1,5 ml of methanol, 0,1 mL 10 percent AlCl3, 0,1 mL Natrium acetate 1 M, and 2,5 mL distillate water were added to a 0.5 mL sample. Previously, incubated for 30 min at room temperature assessed using UV-VIS spectrophotometer at 415 nm. Rutin as a reference, the findings were presented as rutin equivalents (mg RE/g). The formula was used to determine the absorbance of TPC and TFC [9]:

$$\text{TPC or TFC} = c \times V/m \quad (1)$$

c = concentration V= volume of sample (mL); m= mass of sample (gram).

Determination of Sun Protection Factor (SPF)

UV spectrophotometry was used to calculate the SPF [4] method. The substance was dissolved in distilled water at 100, 200, and 300 ppm. The absorbance in a sample concentration was taken between 290 and 320 nm for SPF, 290-315 nm for % of erythema, and 320-370 nm for % of pigmentation. The absorbance data were obtained using a 5 nm triplet interval measurement, accompanied by the Eq.(2) [10].

$$SPF(spectrophotometric) = \mathrm{CF}\, x \sum_{290}^{320} EE\ (\lambda)\ x\ I\ (\lambda)\ x\ Abs\ (\lambda) \tag{2}$$

EE (λ) spectrum of erythemal; I (λ) solar intensity spectrum; Abs (λ) absorbance of the sample; CF correction factor (= 10). The value of EE (λ) x I (λ) is constant, showed in [10].

Determination of Transmission Erythema and Pigmentation

The sample was arranged similarly to SPF's analysis and was evaluated using a UV-Vis spectrophotometer at wavelengths ranging from 292 to 372 nm. The formula is a = compute absorbance (A), Transmission (T) - log T. Then, through the Te = T x Fe formula, the erythema (Te) transmission is computed. Where (Fe) is the erythema's flux, which can be detected at specific wavelengths [11]. The flow value, erythm, which is continued by the sunscreen (Ee), is calculated by the equation, Ee = □ (T x Fe) (T x Fe). The erythm transmission % is calculated by using the following formula:

$$\%\ \text{Transmission erythm} = \frac{\mathrm{Ee}}{\sum \mathrm{Fe}} = \frac{\sum (\mathrm{T} \times \mathrm{Fe})}{\sum \mathrm{Fe}} \tag{3}$$

The absorbance was read at 292-372 nm using the same SPF dosage and erythema. The methodTp= T x Fp, where Fp is the flow of pigmentation with a specific wavelength, is used to calculate pigmentation transmission (TP). calculation Ep = (T x Fp). The pigmentation transmission percent is calculated using the following formula:

$$\%\ \text{Transmission pigmentation} = \frac{\mathrm{Ep}}{\sum \mathrm{Fp}} = \frac{\sum (\mathrm{T} \times \mathrm{Fp})}{\sum \mathrm{Fp}} \tag{4}$$

As shown in Table 1, the sunscreen activity category is next evaluated created on the percent of erythema and pigmentation. The flux of erythema and pigmentation at various wavelengths is shown in [11].

TABLE 1. Table of sunscreens category

Category	Transmission UV (%)	
	Erythema	Pigmentation
Sunblock	<1	3-40
Extra Protection	1-6	42-86
Standard Suntan	6-12	45-86
Fast tanning	10-18	45-86

Determination of Inhibition Tyrosinase

Tyrosinase inhibition was used. [12] With modifications, 500 µL of n-hexane fractions from Averrhoa bilimbi were mixed in 1000 µL of phosphate buffer pH 6.8, and 500 µL of L-tyrosine (2.5 µM) were poured on a phosphate buffer pH 6.8. For 10 minutes, the mixture was incubated in a dark place. At that time, 500 µL of mushrooms tyrosinase solutions (25 KU) was added. The mixture and subsequently kept at room temperature for 30 min. A spectrophotometer was used to assess the absorption at 480 nm. The kojic acid was performed at a 10-20 g/mL concentration and preserved with a similar sample. The complete analytical technique is followed for blank, with all chemicals employed, but no samples are used. The inhibition effect of the N-hexane fraction was analyzed in terms of the inhibitor concentration that resulted in a 50 percent inhibition in enzyme activity (IC50)[13]. The experiment was repeated three times, and the data was analyzed by calculating the percentage of inhibition using the formula [14]:

$$\% \, Inhibition = \frac{(A - B) - (C - D)}{(A - B)} X \, 100 \quad (5)$$

RESULTS AND DISCUSSION

Determination of Total Phenolic Content (TPC)

Folin-Ciocalteu was combined with an N-hexane fraction of Averrhoa bilimbi leaves and gallic acid as a reference, and then placed into a Na_2CO_3 solution, resulting in a blue hue molybdenum-tungsten complex. The phenolic substance only reacts with the Folin-ciocalteu reagent in an alkaline environment, allowing proton separation of the phenolic component captured on phenolic ions and the addition of Na_2CO_3 solutions to the sample. In 120 minutes, the mixture of gallic acid and the Folin-Ciocalteu reagent reached a constant absorbance. The absorbances with the highest measuring sensitivity will be computed using the maximum wavelength produced. 718 nm is the maximum wavelength obtained. The calibration curves equations for gallic acid were found to be (y = 0.0054 x + 0.0325, R = 0.99784). N-hexane fraction total phenolic content: 68.26 8.58 mg GAE/g sample.

According to [7], The TPC ethanolic extract of Averrhoa bilimbi fruits contains 19.8022 1.6696 mg GAE/g, compared to 6.58860.0590 mg GAE/g in the leaves ethanolic extract. In another investigation, the TPC of A. bilimbi was 38.7879 0.9462 mg/g GAE [15]. Phenolic chemicals from plants belong to a family of bioactive components that have garnered significant attention in recent years, mainly owing to their favorable impacts on diet-health interaction in public nutrition[16]. Phenolics are frequent secondary metabolites in plants that have antimutagenic, antioxidant, and anticarcinogenic properties [17]. Mixed solvents have different volumes for extracting the phenolic components; hence, a different solvent with varying polarity leads to diverse outcomes. High polarity solvents may enhance the yield of extracting [18]. Also, varieties of solvent ecological elements such as soil, rain, and sunshine exposure are the principal factors that plant extracts phenolic content. Hence, diverse species of plants display varying total phenolic content [18]. The extract's falling power was seen to rise when the extract's attention was continuously enhanced. These studies propose utilizing their phenols or comparable chemicals by hydrogen donating ability [19].

Determination of Total Flavonoid Content (TFC)

The N-hexane component of Averrhoa bilimbi leaves and rutin standards are mixed with AlCl3 reagents to produce a rutin complex-AlCl3; the solution takes on a brighter yellow hue, indicating a considerable wavelength shift. The addition of sodium acetate aids in the preservation of visible wavelengths (visible). For 30 minutes, the rutin combination with potassium acetate and aluminum chloride reagent was continuously absorbed. 413 nm is the longest wavelength measured. The linear calibration rutin formula was found to be (y = 0.0026 x + 0.018, R = 0.99755). The total flavonoids concentration of fruits ethanolic extract is 112.31±7.22 mg RE/g sample. The finding from this investigation are more significant than the study [9] where is the ethyl acetate fraction of Averrhoa bilimbi fruit SPF (15, 38 ± 1, 3), TPC (56, 87 ± 1, 5212) mg (RE)/g EA) and maximum TFC (24, 99 ± 3, 2895) mg (GAE) / g EA). TFC of an ethanolic extract of Averrhoa bilimbi leaves with 50%, 70%, and 96 percent solvent concentrations were tested at 62.74, 64.81, and 59.1 mg RTE/g extract, respectively, according to other study, while the TPC was recorded as follows: 103.79 mg GAE/g extract; 119.47 mg GAE/g extract; 110.10 mg GAE/g extract [9]**.** A bilimbi contains 1.6666 ± 0.87233 mg/g quercetin equivalents [15].According to [20], the Extraction of Averrhoa bilimbi leaf using 50 percent aqueous methanol yields the maximum antioxidant activity and flavonoid concentration. The maximum TFC (193.3 µg QE/g dry weight), TPC (717.8 µg GAE/g dry weight), and antioxidant activity (77 percent) have disclosed that methanol extract was superior compared to water extract for antioxidant, phenolic, and flavonoid content from Averrhoa bilimbi. According to [21], phenolic substances that contribute to their antioxidant ability are more significant than antioxidant vitamins.

Determination of Sun Protection Factor (SPF)

The SPF in vitro has been tested using the spectrometric approach described by [10]. Considering the UVB area, regarded as the region of most major incidence during the day in which participants have been exposed longer [4]. Figure 1 shows that the SPF value of the N-hexane portion of Averrhoa bilimbi leaves ranges from the least likely (100 ppm) to the full potential (300 ppm). These findings suggest that the active sunscreen chemicals identified on the

leaves of Averrhoa bilimbi are phenolics and other components with linked double bonds that absorb UV radiation in the 290-320 nm range.

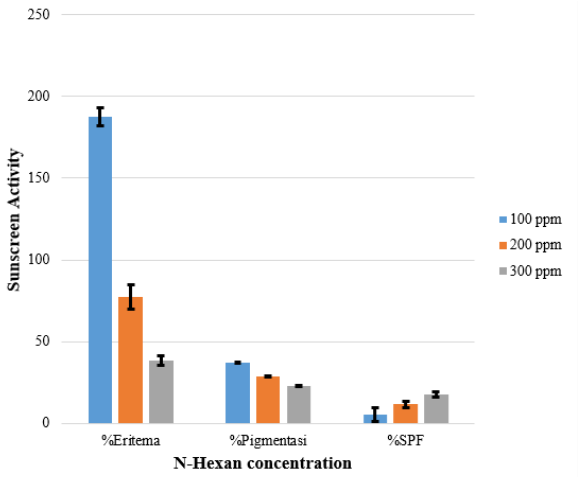

FIGURE 1. In vitro Sunscreen activity of N-Hexane fraction from Averrhoa bilimbi

UV light at a wavelength of 290-320 nm increases inflammation and causes skin to get red or erythematous. Erythema is separated into three phases: skin redness, skin shrinkage, and epidermal cell release—at doses of 100 to 300 ppm, the percentage pigmentation value decreases. The impact, however, is still insufficient to be utilized as a single sunscreen. Only one leaves a 300 ppm threshold, which is displayed in the overall block valuation category. In theory, all the extracts are as effective as sunscreen. This is due to the presence of bioactive chemical groups. Flavonoids and polyphenols are secondary metabolite compounds that can act as sunscreens. To far, about 4,000 flavonoids have been discovered. UV protection is provided by these chemicals in plants [22]. The photoprotective qualities of flavonoids (rutin and quercetin) have long been known. They provide SPF values like homosalate when mixed in oil-in-water emulsions at a concentration of 10% (w/w). In the UVA wavelength, they also provided a significant level of photoprotection. The SPF achieved when combined with titanium dioxide was around 30 [23].

A quantity of 2 mg/cm^2 is utilized for SPF testing since it is more repeatable than a lesser amount of 0.5–1.0 mg/cm^2 [24]. According to [1], natural substances for oral and topical like us polyphones (tannins, flavonoids) potential as sunscreen. The content of phenolic and flavonoid compounds, as well as antioxidant and photoprotective capabilities, were investigated in another study [25].

Determination of Transmission Erythema and Pigmentation

On healthy people, topical use of flavonoids from capers inhibits erythema in multiple ways [26]. Flavonoids (kaempferol, quercetin, and galangin), which are known to be effective antioxidants and have a high UV absorption, were discovered in the same study. Topical antioxidants and DNA repair accelerators are being investigated as possibilities for increasing the photoprotective effects of sunscreens, according to [27]. Topical antioxidants, especially flavonoids, may help to prevent UV-induced skin damage. They are, however, unstable and only spread weakly into the epidermis. Rutin was used as an antioxidant in a sunscreen formulation in a prior study. As a result, its safety and efficacy were investigated [28].

Determination of Inhibition Tyrosinase

It is possible to test for tyrosinase inhibitory activity [12]. In ideal test circumstances, a maximum wavelength of 480 nm, a 30 minute incubation duration, and a 2.5 mM L-tyrosine concentration were used. The temperature is 25-30 °C and the concentration is 25 KU. For each test, sample control solutions were created to compare sample absorption data with and without enzymes. Blank solutions, on the other hand, were created as a corrective factor. Using kojic acid as a reference sample, the Ic50 of Tyrosinase inhibitory activity is 470.9282.31 g/mL, which is higher than the 16.68 g/mL found in kojic acid. The most effective capacity to decrease tyrosinase activities was obligated

by the Standard of kojic acid. Kojic acid ($C_6H_6O_4$), also known as 5-hydroxy-2-(hydroxymethyl)-4-pyrone, is a well-known skin whitening agent or pigmentation inhibitor in both plants and animals. Different solvents have different abilities to inhibit the tyrosinase process in plants [18]. Tyrosinase recognizes polyphenols as substrates. If polyphenols can act as an inhibitor, it depends on the presence and location of another substance. Flavonol inhibitors generally inhibit L-dopa oxidation by tyrosinase through a competitive inhibition mechanism. Copper chelation relies heavily on the 3-hydroxy-4-keto moiety of flavonol molecules [22]. The flavonol glycosides of quercetin or kaempferol were shown to be less effective than their similar aglycones in the same review evaluation of inhibitor strength, quercetin > myricetin > kaempferol >galangin> morin.

CONCLUSION

This study reveals that the sunscreen and inhibitor tyrosinase activity reported on N-hexane, the leaves of Averrhoa bilimbi, are suspected of flavonoid and phenolic substances. Hence, more research is required to explain the extended employment of sun protection to the topical dose form.

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