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Original Article

Antioxidant Activity and Tyrosinase Inhibition of Avocado (*Persea americana* Mill.) Leaves and Seeds Extracts

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ABSTRACT

Background: Ultraviolet radiation (UVR) can stimulate melanin production in the skin, leading to hyperpigmentation. One way to reduce melanin formation is by inhibiting the enzyme tyrosinase. Often discarded as waste, avocado leaves and seeds are rich in flavonoids with potential tyrosinase-inhibiting properties. **Objectives:** This study aimed to perform phytochemical screening, measure antioxidant and tyrosinase inhibitor activities, and explore the correlation between free radical scavenging and tyrosinase inhibition in ethanol extracts of young avocado leaves (EAL(O)) and old avocado leaves (EAL(Y)), as well as the ethyl acetate extract of avocado seeds (EAS). **Methods:** Antioxidant activity was assessed using the *2,2-Diphennyl-1-picrylhydrazyl* (DPPH) method, while tyrosinase inhibition was evaluated in vitro with mushroom tyrosinase and L-DOPA as a substrate. **Results:** The results of the antioxidant activity test using the DPPH method yielded IC_{50} values for EAL(Y), EAL(O), and EAS of 17.9±0.15 µg/mL, 15.33±0.02 µg/mL, and 745.66±6.08 µg/mL, respectively. Tyrosinase inhibition tests indicated that EAL(Y), EAL(O), and EAS could significantly inhibit tyrosinase activity (P <0.01), with IC_{50} values of 10133.26±140.12 µg/mL, 8496.20±155.06 µg/mL, and 48375.8±553.06 µg/mL, respectively. The correlation coefficients between tyrosinase inhibition and antioxidant activity for EAL(Y), EAL(O), and EAS were r = 0.955, r = 0.947, and r = 0.991, respectively. **Conclusion:** These findings suggest that EAL(Y), EAL(O), and EAS may be potential natural skin-whitening agents due to their flavonoid content and antioxidant and anti-tyrosinase properties.
Key words: *Persea americana* Mill; tyrosinase inhibition; DPPH; L-DOPA.

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INTRODUCTION

Repeated exposure to ultraviolet (UV) radiation can activate melanin synthesis, increasing melanin content in human skin[1]. Melanin plays a vital role in determining skin color, while melanogenesis serves as a protective mechanism against skin damage caused by UV exposure[2]. Excessive melanin accumulation in various parts of the skin can lead to abnormal hyperpigmentation, commonly associated with conditions such as melasma, freckles, post-inflammatory hyperpigmentation, ephelides, lentigo, nevus, and melanoma[3].

The primary enzyme responsible for melanin synthesis is tyrosinase, which participates in two catalytic reactions. The process begins with the oxidation of the amino acid L-tyrosine to form 3,4-dihydroxyphenylalanine (L-DOPA), which is then oxidized to dopaquinone, with both reactions facilitated by tyrosinase. Following this, dopaquinone is converted into dopachrome, which is then further changed into 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), leading to the production of melanin[3]. One way to prevent melanin formation is by inhibiting the activity of the tyrosinase enzyme to avoid hyperpigmentation[4].

Many well-known tyrosinase inhibitors exist, such as hydroquinone, arbutin, kojic acid, azelaic acid, and L-ascorbic acid[1]. Although hydroquinone is the most popular agent for treating hyperpigmentation because it can inhibit tyrosinase activity, it has numerous side effects, including skin irritation, toxicity, mutagenicity, carcinogenicity, and exogenous ochronosis. This has led to discontinuing many whitening agents due to safety concerns, making it essential to seek new compounds with better effects on tyrosinase inhibition and fewer side effects[2]. As a result, numerous researchers concentrate on using botanical and natural substances as safe and effective alternatives for depigmenting agents[1].

The avocado plant (*Persea americana* Mill.), which belongs to the *Lauraceae* family, is widely cultivated in subtropical and tropical regions[5,6] and grows abundantly in the highlands of Indonesia[7]. The flesh of the avocado fruit is consumed, while the Indonesian population uses its seeds and leaves as alternative herbal medicine. Avocado leaves, often regarded as waste or organic trash[6], contain active flavonoid compounds, specifically quercetin.[8] Previous research has found that avocado seeds contain catechin, a flavonoid metabolite[9]. Flavonoids have the potential to inhibit tyrosinase[10,11] by forming chelates with copper ions, thereby irreversibly deactivating tyrosinase by generating interactions between flavonoids, copper ions, and the tyrosinase catalytic domain[12], thus acting as a skin-whitening agent[9].

This study seeks to evaluate the phytochemical composition, free radical scavenging ability, and anti-tyrosinase activity of ethanol extracts from young avocado leaves (EAL(Y)) and old avocado leaves (EAL(O)), in addition to the ethyl acetate extract from avocado seeds (EAS). The results of this research are anticipated to contribute to the application and development of natural skin-whitening agents.

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MATERIAL AND METHODS

Research design

The research design is a laboratory experiment with a completely randomized design (CRD) that is comparative. The research was conducted at the School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, Indonesia, and the Laboratory of the Tropical Biopharmaca Research Center (TropBRC) IPB, Bogor, Indonesia.

Extraction

The extraction method uses maceration[9]. The solvent used was 96% ethanol (Brataco, Jakarta, Indonesia) for avocado leaves and ethyl acetate (Brataco, Jakarta, Indonesia) for avocado seeds for three days (3x24 hours), changing the solvent daily. The filtrate obtained was evaporated using a rotary evaporator at 60°C until a concentrated extract was obtained.

Phytochemical Screening

The phytochemical screening procedure follows the method by Reji and Rexin (2013)[13]. The aim was to identify the presence of biochemicals, including flavonoids, alkaloids, tannins, saponins, steroids, and triterpenoids.

2,2-Diphennyl-1-picrylhydrazyl (DPPH) Assay

The DPPH Assay antioxidant test method against DPPH free radicals used in this research is a modification of several procedures reported by Sutriningsih et al. (2017)[14].

Tyrosinase Inhibitor Assay

A volume of 70 µL of the extracts at various concentrations was added to a microplate, followed by 30 µL of mushroom tyrosinase (Sigma-Aldrich, USA). The plate was incubated at 37°C for 5 minutes. After that, 110 µL of the substrate L-DOPA (Sigma-Aldrich, USA) at 12 µM was added to the microplate and kept in the incubator for 30 minutes. The absorbance values for each microplate well were measured using a multi-well reader at a wavelength of 510 nm. The percentage of tyrosinase activity was calculated using the formula:

Inhibition (%) = ((Absorbance of Blank - Absorbance of Sample) / Absorbance of Blank) x 100%

DATA ANALYSIS

All experimental results are presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted using one-way Analysis of Variance (ANOVA) and was followed by Dunnett's post-hoc test. *P*-values less than (P<0,01) were considered statistically significant. Pearson's method assessed the correlation between free radical scavenging activity and tyrosinase inhibition.

RESULTS

Phytochemical Screening and DPPH Examination

Phytochemical screening was conducted to identify and analyze the secondary metabolites in EAL(Y), EAL(O), and EAS. The outcomes of the phytochemical analysis performed in this study are shown in Table 1. The ability to scavenge free radicals from EAL(Y), EAL(O), and EAS was detected using the DPPH method. The results indicated that the free radical scavenging activity depended on the dose. At a concentration of 100 µg/mL, EAL(Y) exhibited a free radical scavenging activity of 89.07%, EAL(O) at 100 µg/mL showed an activity of 89.28%, and EAS at a concentration of 1000 µg/mL demonstrated an activity of 62.62% (Figure 1).The IC_{50} values were determined from the linear regression curve between the percentage of absorption inhibition and the concentrations of EAL(Y), EAL(O), EAS, and vitamin C as the positive control. The results are shown in Table 2.

Table 1. Phytochemical screening of EAL(Y), EAL(O), and EAS.

Phytochemical compound	EAL(Y)	EAL(O)	EAS
Flavonoids	+	+	+
Alkaloids	+	-	-
Tannins	+	+	-
Saponins	+	+	+
Quinone	-	-	+
Steroids	+	+	-
Triterpenoids	-	-	+

EAL(Y); Ethanol Extract of Young Avocado Leaf ; EAL(O); Ethanol Extract of Old Avocado Leaf; EAS: Extract Ethyl Acetate of Avocado Seeds

Table 2. Linear regression equations and 50% inhibitory concentration (IC_{50}) values (g/mL) of EAL(Y), EAL(O), and EAS.

Sample	Equation	R^2	IC_{50} (µg/mL)
EAL(Y)	y = 2,5296x + 4,2784	0,9989	17,9±0,15
EAL(O)	y= 2,8414x + 6,4948	0,9971	15,33±0,02
EAS	y = 0,0587x + 6,4219	0,9934	745,66±6,08
Vitamin C	y = 7,3488x + 1,1954	0,9997	6,61±0,03

EAL(Y); Ethanol Extract of Young Avocado Leaf;
EAL(O); Ethanol Extract of Old Avocado Leaf;
EAS: Extract Ethyl Acetate of Avocado Seeds

Table 3. The linear regression equation and IC_{50} value (µg/mL) of inhibition tyrosinase.

Sample	Equation	R^2	IC_{50} (µg/mL)
EAL(Y)	y = 14,318ln(x) - 82,249	0,9744	10133,26±140,12
EAL(O)	y=14,879ln(x) - 84,862	0,9845	8496,20±155,06
EAS	y = 16,2ln(x) - 124,9	0,9837	48375,8±553,06
KA	y=14,115ln(x) + 14,935	0,9841	11,86±0,13

EAL(Y); Ethanol Extract of Young Avocado Leaf; EAL(O); Ethanol Extract of Old Avocado Leaf; EAS: Extract Ethyl Acetate of Avocado Seeds; KA: Kojic Acid

Tyrosinase Inhibitory Activity

The tyrosinase inhibitory activity of EAL(Y), EAL(O), and EAS was determined using a mushroom tyrosinase assay with L-DOPA as the substrate. The results showed that EAL(Y) and EAL(O) extracts at concentrations of 1562.5–100.000 µg/mL, as well as EAS at 3125–50.000 µg/mL, significantly inhibited mushroom tyrosinase activity in a dose-dependent manner (P<0.01) (Figure 2), compared to the negative control (untreated with EAL(Y), EAL(O), and EAS). The results of the tyrosinase inhibitory activity analysis are shown in Table 3.

Correlation between Mushroom Tyrosinase Inhibition and Free Radical Scavenging Activity

In this study, there was a high correlation between mushroom tyrosinase inhibition and DPPH activity, as shown by the correlation coefficients for tyrosinase inhibition and radical scavenging activity of EAL(Y), EAL(O), and EAS, which were r = 0.955, r = 0.947, and r = 0.991, respectively (Figure 3). These findings suggest that the higher DPPH radical scavenging activity of EAL(Y), EAL(O), and EAS also leads to increased inhibition of tyrosinase activity.

DISCUSSION

The results of the phytochemical tests on EAL in this study align with those found in previous research, which reported the presence of flavonoids, alkaloids, tannins, saponins, and steroids[15]. In this study, the phytochemical test results for EAS are consistent with previous research

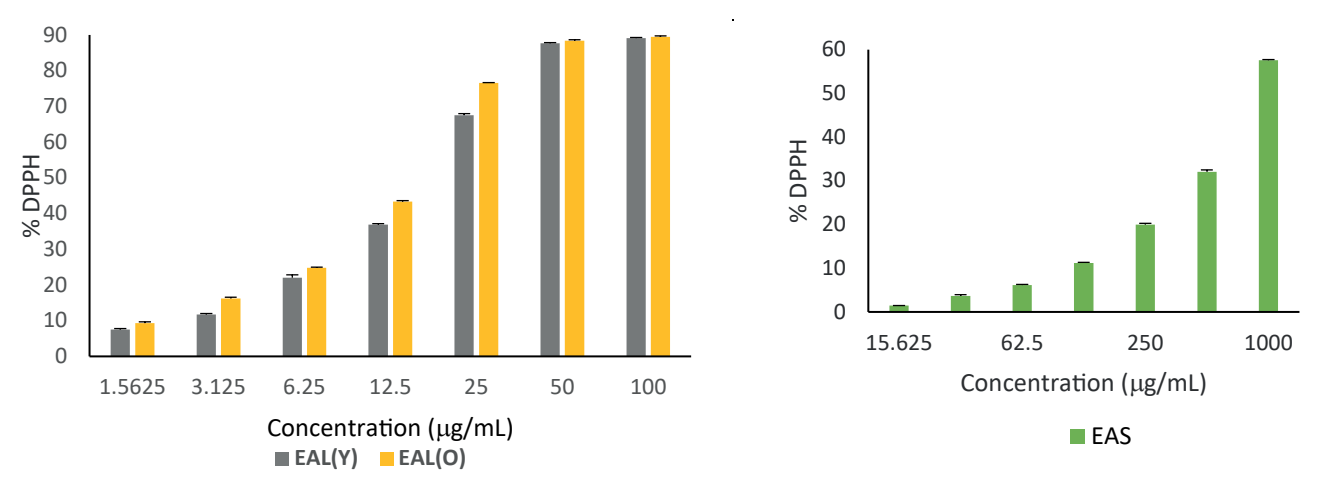

Figure 1. The scavenging activity of EAS determined by the DPPH assay. Each bar represents the mean ± standard deviation of three replicates.

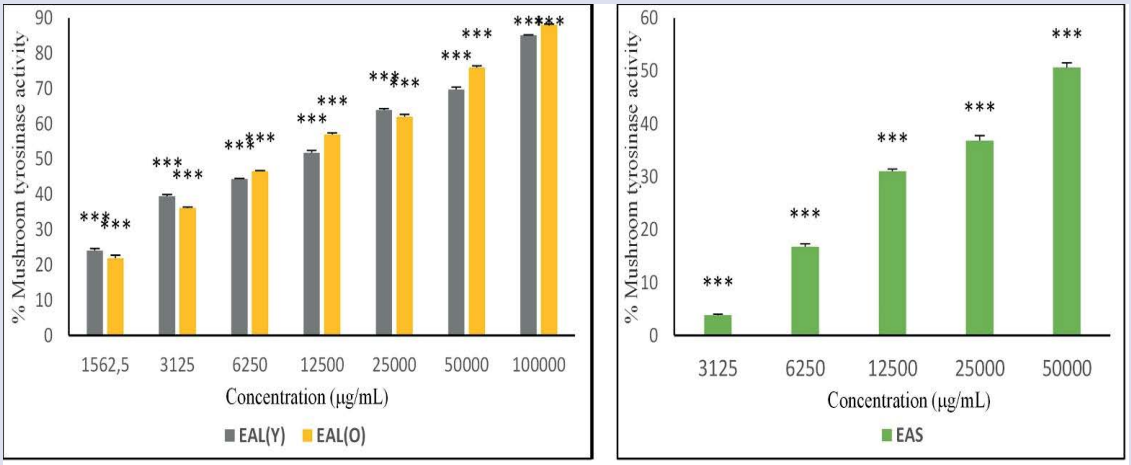

Figure 2. The effect of extracts EAL(Y), EAL(O), and EAS on mushroom tyrosinase activity. Data are presented as the mean ± SEM of three independent replicates. ***P < 0.01 compared to the control group (untreated).

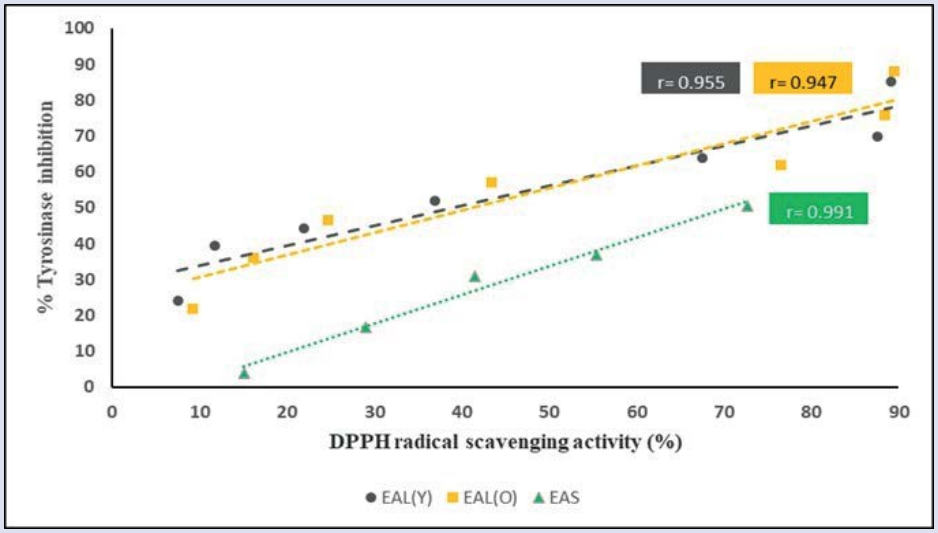

Figure 3. Correlation between mushroom tyrosinase inhibition and free radical scavenging activity EAL(Y), EAL(O), and EAS.

findings, which reported the presence of flavonoid[16], saponins[16], quinones[17], and triterpenoids[16,17]. Among these, flavonoids are the most likely contributors to both antioxidant and tyrosinase inhibitory activities observed in this study. Flavonoids are well-known for their ability to scavenge free radicals by donating electrons or hydrogen atoms, thereby stabilizing reactive oxygen species (ROS), which are closely associated with oxidative stress-induced melanogenesis[3,10]. Flavonoids can also inhibit tyrosinase activity through direct interaction with the enzyme. Their polyphenolic structure allows them to chelate copper ions at the tyrosinase active site, thus blocking the catalytic process that converts L-tyrosine to dopaquinone[12]. Specific hydroxyl substitutions on the flavonoid backbone are associated with stronger tyrosinase inhibition, as previously demonstrated in structure-activity relationship studies[11]. The strong correlation observed in this study between DPPH scavenging and tyrosinase inhibition further supports the hypothesis that flavonoids are major bioactive contributors. Saponins, which were also present in the extracts, may enhance bioavailability by affecting membrane permeability and could provide auxiliary antioxidant effects[18].

Tannins, detected in the leaf extracts, are polyphenols capable of metal ion chelation and may contribute to both antioxidant and enzyme-inhibitory effects[19]. Although not the primary focus of this study, alkaloids, quinones, and triterpenoids may also contribute synergistically to the overall biological activity. These compounds have been reported in the literature to possess various pharmacological properties, including antioxidant and anti-tyrosinase effects[20].

Therefore, the biological effects observed in this study are likely the result of a synergistic interplay of multiple phytochemicals, with flavonoids acting as the principal agents, supported by saponins, tannins, and possibly other constituents. Further isolation and mechanistic studies are needed to confirm the individual roles and efficacy of these compounds. Several studies indicate that the content of secondary metabolic compounds, such as flavonoids, which function as antioxidants in plants, can vary by region. These variations may be due to environmental factors, such as light, temperature, pH, and altitude, which can affect the chemical composition of a plant[21].

Another factor that can influence the production of bioactive compounds in avocado leaves is the age of the leaves. In this study, EAL(O) exhibited higher antioxidant content than EAL(Y). This is because older leaves have more mature structural components, containing sufficient nutrients and chlorophyll to capture sunlight, leading to a higher rate of photosynthesis. The higher photosynthesis rate is associated with greater production of secondary metabolites, including flavonoids, which are more abundant in older leaves than younger ones. Carbon compounds resulting from photosynthesis serve as a source for the formation of secondary metabolites[6].

The use of different solvents, 96% ethanol for avocado leaf extraction and ethyl acetate for avocado seed extraction, was based on prior studies that optimized extraction conditions specific to each plant part. A wide variety of polar to moderately polar phytochemicals, especially flavonoids, tannins, alkaloids, and phenolics, can be extracted from leaves using ethanol, a polar protic solvent[22]

It is well known that avocado leaves are rich in these polar chemicals, and earlier studies have shown that 96% ethanol efficiently extracts anti-tyrosinase and antioxidant components from avocado leaf material.[6] Conversely, ethyl acetate is a semi-polar solvent that has been chosen especially for avocado seeds since previous research has shown that it effectively extracts catechins and triterpenoids from seed matrices[17].

Avocado seed ethyl acetate fractions include catechin, a crucial component with documented anti-tyrosinase activity[9]. The purpose of this study was to describe the potential of each extract in its most active and extractable form rather than to directly compare the potency of each extract across plant parts, even though it is recognized that solvent polarity affects the metabolite profile and that using different solvents may introduce variability. In order to optimize the recovery of physiologically active chemicals from each unique matrix, various solvents were purposefully selected. To ensure fairness in interpretation, the results were presented and discussed separately for each extract type. Moreover, all extracts were subjected to the same biological assays under uniform conditions, allowing for consistent comparison of their functional properties, such as antioxidant and tyrosinase inhibitory activities.

The use of tyrosinase enzymes has been widely applied in the study of natural substances as tyrosinase inhibitors in vitro, with results that closely resemble the effect of tyrosinase inhibition in human skin. Although in vitro results are not always visible on human skin, this method is commonly used in melanogenesis research, particularly as an initial step to identify the potential activity of depigmentation agents. The tyrosinase inhibition test uses mushroom tyrosinase without cultured cells[23]. Tyrosinase inhibitor activity is assessed through an in vitro tyrosinase enzyme inhibition test using L-DOPA as the substrate[1]. The principle of the in vitro method is based on the production of dopachrome, the result of L-DOPA oxidation by the tyrosinase enzyme. Skin-whitening compounds compete with L-DOPA to bind to the tyrosinase enzyme. This competition reduces the amount of dopachrome produced, enabling the calculation of the compound's inhibition activity. The dopachrome produced ranges in colour from deep orange to red[9]. Its activity is evaluated by measuring dopachrome formation, which can be quantified spectrophotometrically to determine the IC_{50} value[23]. Results from previous research that tested tyrosinase activity using mushroom tyrosinase with a different method yielded an IC_{50} value of 93.02±1.98 µg/mL[9]. We believe that the differences in results may be due to variations in enzyme and substrate concentrations and the fact that tyrosinase enzymes are susceptible to environmental conditions such as pH and temperature.

These results indicate a first step in the screening of natural compounds with possible anti-tyrosinase activity, even if the IC_{50} values for tyrosinase inhibition of EAL(Y), EAL(O), and EAS are comparatively high in comparison to kojic acid. The study's assay was based on the non-cellular model of mushroom tyrosinase, which is frequently utilized in early-stage research but falls short of accurately simulating the intricacy of human melanogenesis in vivo[23]. Nevertheless, a strong correlation was observed between antioxidant activity and tyrosinase inhibition ($r > 0.94$), indicating that these extracts contain bioactive compounds with potential dual action.

The extracts' high IC_{50} values imply that their effectiveness for direct cosmetic application may be limited in their crude form. But it's crucial to remember that biological factors like bioavailability, skin penetration, metabolism, and formulation can affect in vitro IC_{50} values, which means they don't always correspond to in vivo efficacy[24,25]. Additionally, the extracts' inhibitory effect may be increased by further fractionation and purification or by combining them with other substances.[3] Thus, this study is only a first step, and more research utilizing cellular models like melanocytes or melanoma cells is required to more accurately determine biological relevance[2].

This study found a positive correlation between free radical scavenging activity and mushroom tyrosinase inhibition. Our research, which utilized mushroom tyrosinase enzyme, demonstrated that increasing the concentrations of EAL(Y), EAL(O), and EAS resulted in a higher percentage of tyrosinase enzyme inhibition (Figure 3). The flavonoid content in EAL(Y), EAL(O), and EAS can chelate metal ions, thereby preventing redox reactions that produce free radicals. The flavonoids in EAL(Y), EAL(O), and EAS play a role in inhibiting tyrosinase activity in the melanogenesis process. The phytochemical content in EAL(Y), EAL(O), and EAS, particularly flavonoids, is likely to protect the skin from free radicals and prevent hyperpigmentation[26].

However, there are no reports regarding the correlation between mushroom tyrosinase inhibition and free radical scavenging activity for EAL(Y), EAL(O), and EAS. The anti-tyrosinase activity of EAL(Y), EAL(O), and EAS is still deficient compared to KA, but to address hyperpigmentation, combining different active ingredients in the same formulation can be effective[24]. Our findings suggest that EAL(Y), EAL(O), and EAS contain flavonoids, exhibit antioxidant activity, and have anti-tyrosinase properties, making them potential candidates for development as alternative skin-whitening agents. These could be used in combination with other whitening agents for reducing hyperpigmentation.

CONCLUSION

Based on in vitro testing, EAL(Y), EAL(O), and EAS can inhibit melanin synthesis and tyrosinase activity through their antioxidant properties, making them potential alternatives for natural depigmentation agents. This study provides preliminary evidence of the extract's tyrosinase inhibitory activity based on the mushroom tyrosinase assay, which serves only as an initial insight. Further investigations using melanocyte or B16F10 cell models are planned to understand better the in vivo relevance, particularly given the current absence of cellular or animal models. Future studies should also include gene and protein expression analyses using RT-PCR/qPCR and Western blot to determine which signaling pathways are involved in the inhibition of melanogenesis.

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