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Research Article

Cytotoxicity of Ethyl Acetate Extract of Avocado Seeds (*Persea americana* Mill.) on Mouse Melanoma B16F10 Cell Line

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ABSTRACT

Artificial whitening agents available in the market can cause damage to melanocytes; therefore, a safe and naturally derived skin-whitening agent is needed. Avocado seeds (*Persea americana* Mill.) are often discarded as waste but possess numerous health benefits. One of the compounds in avocado seeds is catechin, a flavonoid metabolite. This compound has been reported to exhibit antioxidant activity and inhibit tyrosinase to prevent melanin formation, making it suitable for skin-whitening applications. This study aimed to conduct phytochemical screening, assess antioxidant activity using the DPPH method, analyze catechin content using HPLC, and perform cell viability tests using the MTT method from the ethyl acetate extract of avocado seeds. Phytochemical screening has revealed the presence of flavonoids, saponins, and steroids. The DPPH assay yielded an IC_{50} value of 89.47±0.73 ppm, indicating an intense antioxidant activity, and HPLC detected 0.09% catechin. The MTT test results yielded cell viability percentages ranging from 80-100% at test concentrations of 0.75-25 ppm, with an IC_{50} value of 61.7 ppm. In conclusion, our results indicate that the ethyl acetate extract from avocado seeds affects cell viability without toxicity, warranting further testing for tyrosinase inhibition in the mouse melanoma B16F10 cell line.

1. Introduction

Melanin is essential for determining skin color, and melanogenesis is a defense mechanism against skin damage caused by ultraviolet (UV) light (Hwang *et al.* 2022). Melanin is synthesized in melanosomes within melanocytes found in the epidermis (Choi *et al.* 2022). The accumulation of excessive melanin in various parts of the skin can result in abnormal hyperpigmentation, with commonly complicated conditions including melasma, freckles, post-inflammatory hyperpigmentation, ephelides, lentigo, nevi, and melanoma (El-Nashar *et al.* 2021).

Tyrosinase is the primary enzyme involved in melanin synthesis (Hindritiani *et al.* 2013; El-Nashar *et al.* 2021; Hushcha *et al.* 2021), and is involved in two catalytic reactions. First, through hydroxylation, tyrosine is converted into 3,4-dihydroxy-L-phenylalanine (L-DOPA) by the monophenolase activity of tyrosinase. Second, through oxidation, L-DOPA is converted into DOPA-quinone by the diphenolase activity of tyrosinase (Hwang *et al.* 2022). Preventing melanin formation by inhibiting tyrosinase enzyme activity is an approach to avoid hyperpigmentation (Goenka & Toussaint 2020).

Efforts to reduce or inhibit melanin production are crucial to prevent pigmentation and achieve whitening effects (Choi *et al.* 2022). Whitening agents can be derived from synthetic or natural sources (Hindritiani *et al.* 2013; Widyastuti *et al.* 2023). Conventional whitening agents available on the market include hydroquinone, kojic acid, and arbutin (Hwang *et al.* 2022). Continuous use of hydroquinone can lead to leukoderma and

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exogenous ochronosis (Widyastuti *et al.* 2023). This has resulted in the discontinuation of many whitening agents owing to safety concerns, highlighting the importance of searching for new compounds with better inhibitory effects on tyrosinase activity and fewer side effects (Hwang *et al.* 2022). Naturally occurring pigmentation agents are typically herbal or phytochemical compounds that have been tested as depigmenting agents and are considered safer (Widyastuti *et al.* 2023).

Avocado (*Persea americana* Mill.) originated in Central America and Mexico and is commonly known as the avocado (Castro-López *et al.* 2019; Kemit *et al.* 2019). This plant is extensively cultivated in subtropical and tropical regions (Castro-López *et al.* 2019) and is also prevalent in the highlands of Indonesia (Kemit *et al.* 2019). In addition to being consumed as a fruit, various parts of the avocado, including the leaves and seeds, are recognized for their beneficial health properties (Castro-López *et al.* 2019; Kemit *et al.* 2019). Previous research has found that catechin, a flavonoid metabolite, is present in avocado seeds (Laksmiani *et al.* 2020). This compound contains antioxidants and has the potential to inhibit tyrosinase (Jakimiuk *et al.* 2022) by forming chelates with copper ions, thereby irreversibly deactivating tyrosinase via interactions between flavonoids and copper ions in the catalytic domain of tyrosinase (Kim *et al.* 2004; Laksmiani and Nugraha 2019).

The cell viability was defined as the number of healthy cells in each sample. Viability tests were performed to assess the impact of the developed materials on cells. Cell viability measurements are crucial in all cell culture studies (Kamiloglu *et al.* 2020). Viability was calculated by determining the number of viable cells (Pascayantri *et al.* 2021). A commonly used method for cytotoxicity testing is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Kamiloglu *et al.* 2020). However, this study is the first to establish whether EAS can be investigated in mouse melanoma B16F10 cells. In cell studies, the tested materials must be proven non-toxic to cells.

This study aimed to determine the IC_{50} values and cell viability percentage of ethyl acetate extract from avocado seeds (EAS) against the mouse melanoma B16F10 cell line using the MTT assay. Additionally, we aimed to measure the correlation levels and concentrations of the test substances as preliminary data to establish the optimal concentration of EAS that can inhibit tyrosinase.

2. Materials and Methods

2.1. Materials

The raw material used was avocado seed simplicia obtained from The Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI) Bogor, Indonesia. Ethyl acetate (Brataco, Jakarta, Indonesia) was used as the solvent for extraction. DPPH was performed using 2.2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich), and ethanol was used for the HPLC test. (Merck), and methanol (MeOH) (Merck) were used. Primate Research Center Animal Studies, IPB University, Bogor, Indonesia, provided mouse melanoma B16F10 cultured cells (ATCC CRL 6475/USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco/USA), 10% Fetal Bovine Serum (FBS, Hyclone/USA), penicillin-streptomycin 1% (Invitrogen/USA), phosphate-buffered saline (PBS, Gibco/USA), trypsin 0.25% (Gibco/USA), dimethyl sulfoxide (DMSO, Sigma/USA), and 3-(4.5-dimethyl-2-thiazolyl)-2.5-diphenyl-2H-tetrazolium bromide (MTT, Sigma/USA).

2.2. Extraction

The extraction method used the maceration technique described by Laksmiani *et al.* (2020). Ethyl acetate was used as the solvent for 3 days (3 × 24 h), with daily solvent replacement. The ratio of crude material to solvent used was 1:10. The filtrate was evaporated using a rotary evaporator at 60°C until a concentrated extract was obtained.

2.3. Phytochemical Screening

Phytochemical screening was performed as described by Reji and Rexin (2013). Phytochemical screening was performed using EAS. This study aimed to identify biochemical compounds, including alkaloids, flavonoids, tannins, saponins, steroids, and triterpenoids.

2.4. DPPH Assay

The EAS solution and vitamin C control were each weighed to 10 mg, dissolved in 1 ml of dimethyl sulfoxide (DMSO), and then diluted to 20 ppm by adding ethanol pro analyst (p. a.). Each sample was then dispensed into a microplate at 100 µL per well. Ethanol (100 µL) was added to the negative control, whereas for replicates 1 and 2, 100 µL DPPH solution was added. The blank solutions for replicates 1 and 2 consisted of 100 µL of ethanol per 100 µL of DPPH,

whereas the negative control contained 200 µL of ethanol. The blank solution, vitamin C, and samples were incubated at 37°C for 30 min and their absorbance was measured at 517 nm using a spectrophotometer (Bio-Rad) (Sutriningsih and Astuti 2017).

2.5. Determination of Catechin Levels with HPLC

Sample preparation was initiated by heating 10 ml 70% MeOH at 70°C for 30 min. Subsequently, 0.1 g) was weighed and combined with 8 ml 70% MeOH. The mixture was heated in a water bath for 10 min and vortexed every 5 min. The solution was placed into a 10 ml flask, diluted from 1 to 5 ml with a stabilizing solution, filtered through a 0.45 nm filter, and injected 10 µL into the system (Hitachi) (Angraini and Desmaniar 2020).

2.6. Cell Viability Test Using the MTT Method

Cell viability was assessed using the MTT method developed by Mosmann (1983). This study was conducted in a class IIA biological safety cabinet (NuAire). A 96-well plate were filled in B16F10 cells at a density of 5 × 103 cells/well. Cells reaching 50% confluency (18-20 hours) were treated with 100 µL/well of the bioactive compound, according to their respective concentrations, while the control cells received no treatment. The cells were then incubated for 48 h at 37°C and 5% CO_2. MTT solution (50 µg/well) was added to each well, and the plate was further incubated for four hours at 37°C and 5% CO_2. The supernatant was discarded, and the formed formazan crystals were dissolved in 96% ethanol. Absorbance was recorded using a microplate reader at a wavelength of 595 nm. Optical density (OD) data obtained from the measurements were used to calculate the percentage of cell viability resulting from the treatment. Cell viability was calculated using the MTT assay (Kamiloglou *et al.* 2020):

$$\% \text{ Cell viability} = \frac{\left(\begin{matrix}\text{Sample}\\ \text{absorbance}\end{matrix} - \begin{matrix}\text{Blank}\\ \text{absorbance}\end{matrix}\right)}{\left(\begin{matrix}\text{Control}\\ \text{absorbance}\end{matrix} - \begin{matrix}\text{Blank}\\ \text{absorbance}\end{matrix}\right)} \times 100$$

2.7. Data Analysis

The viability percentage of cells was analyzed using linear regression equations to calculate the IC_{50} value.

3. Results

Phytochemical screening was conducted to identify and analyze the secondary metabolites in the EAS. The results of the phytochemical analysis are presented in Table 1, indicating the presence of flavonoids, saponins, and steroids.

The EAS antioxidant activity test results in this study using the DPPH method can be observed through the measurement of IC_{50}, calculated from the linear regression curve between the percentage of absorption inhibition and the EAS concentration (Figure 1). The IC_{50} value was obtained from the equation y = 21.342–46.149, where x represents the IC_{50} value and y is equal to 50. The DPPH test results for EAS showed an IC_{50} value of 89.47±0.73 ppm, indicating that EAS exhibited strong antioxidant capacity.

Figure 2 shows the chromatogram of a 100 ppm catechin standard solution (injection volume 10 µL) under the operating conditions listed in Table 2. Figure 3 shows a catechin chromatogram of the EAS sample. The measurement results for the sample indicated a catechin content of 0.09% in EAS with a retention time of 5.069 min.

The results of the correlation measurement between absorbance and EAS concentration against the mouse melanoma B16F10 cell line using the MTT method are shown in Table 3. The correlation between EAS and absorbance was -0.97369, indicating a strong relationship between absorbance and concentration. A negative sign indicates an inverse relationship, meaning that as the concentration increases, the absorbance decreases. The decrease in the absorbance of the treated cells indicated the presence of cytotoxicity.

Table 1. Phytochemical test results of EAS

Compound name		Test results
Flavonoid		Positives
Alkaloid	Wagner	Negatives
	Mayer	Negatives
	Dragendorf	Negatives
Tanin		Negatives
Saponin		Positives
Quinon		Negatives
Steroid		Positives
Triterpenoid		Negatives

MTT assays were conducted using various concentrations of EAS, namely 0.75, 1.5, 3.125, 6.25, 12.5, 25, 50, and 100 ppm, at a wavelength of 595 nm. The calculation results indicated that the formed graph was nonlinear (Figure 4). From the graph, it can be observed that cell viability tended to increase with decreasing dosage. The highest cell viability was obtained at a dose of 0.75 ppm (103.91%), and the lowest was observed at 100 ppm (6.79%). The toxicity tended to decrease with decreasing dose concentration.

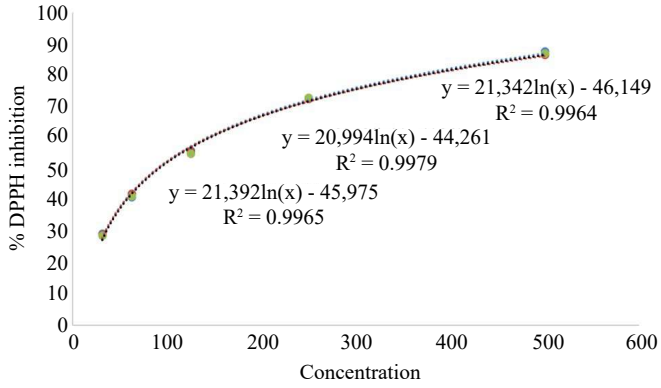

Figure 1. Concentration-inhibition relationship in the antioxidant activity test of EAS using the DPPH method

One method for assessing cell viability is to determine the IC_{50} parameter, which indicates the potential toxicity of a test compound. The IC_{50} value was used to determine the maximum concentration of the test compounds. Based on the polynomial regression equation for cell viability from the EAS, which is $y = 0.8705x^4 - 4.4602x^3 + 5.9904x^2 + 3.3453x - 3.81$ with $R^2 = 0.9833$, the obtained IC_{50} value is 61.07 ppm, as

Table 2. EAS analysis condition

Column	Zorbax eclipse C-18 (8)
Mobile phase	9% acetonitrile, 2% acetic acid, EDTA 20 ppm
Flow rate	1.0 ml/minutes
Detector	Diode array detector
Wavelength	278 nm

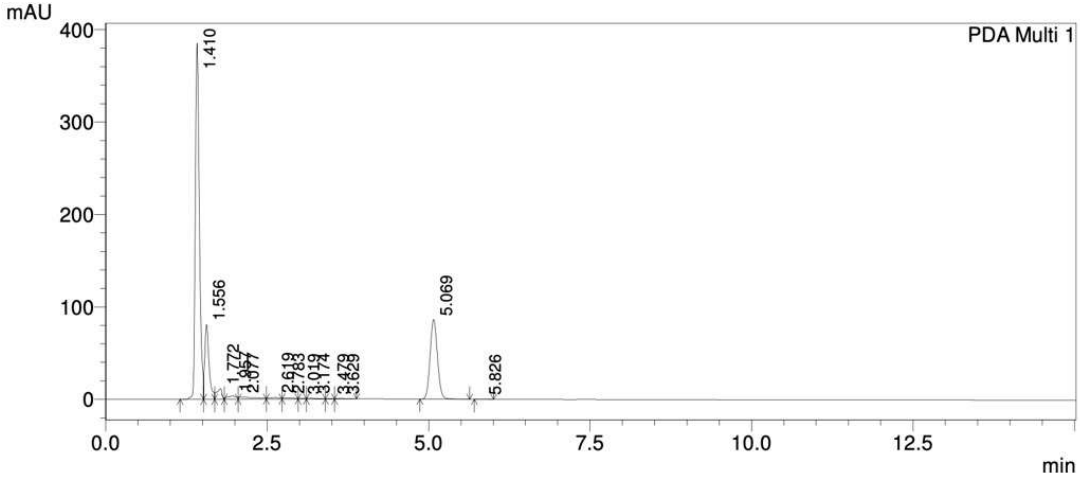

Figure 2. Chromatogram of the 100 ppm catechin standard at 278 nm

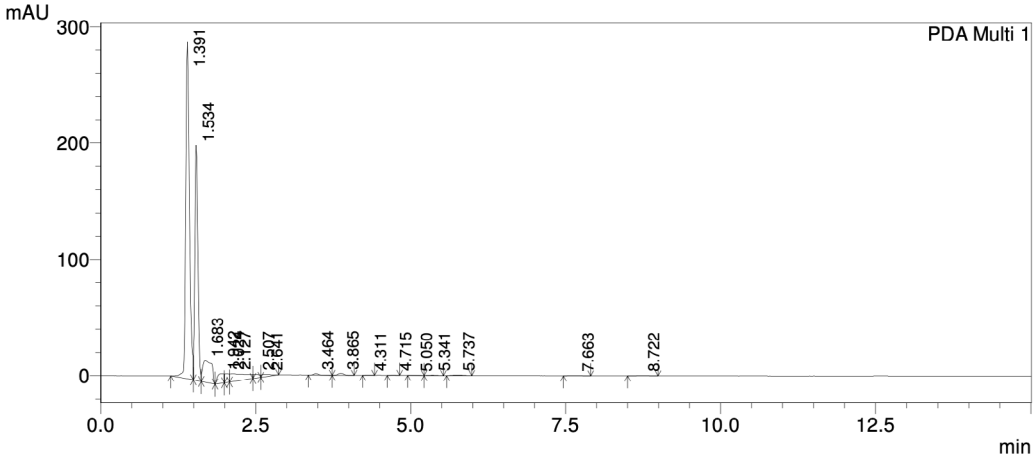

Figure 3. Chromatogram of EAS at 278 nm

Table 3. Correlation between absorbance and EAS concentration

Concentration (%)	Absorbance			Average	Correlation
100	0.079	0.038	0.048	0.550	
50	0.548	0.392	0.439	0.512	
25	0.766	0.756	0.782	0.768	
12.25	0.782	0.658	0.715	0.718	
6.25	0.774	0.772	0.771	0.772	-0.97369
3.125	0.721	0.798	0.852	0.790	
1.5	0.856	0.828	0.805	0.830	
0.75	0.824	0.863	0.837	0.841	
Kontrol	0.825	0.807	0.797	0.809	

shown in Figure 5. The maximum concentration to achieve 50% viability was 61.07 ppm, whereas therapy with 100 ppm reduced cell viability by more than 50%. This indicated that a concentration of 100 ppm was cytotoxic to the mouse melanoma B16F10 cell line.

In the nonparametric T-test of EAS, a concentration of 0.75 ppm was chosen for the subsequent process as it exhibited inhibition values not significantly different from the control (100% viability) at a significance level

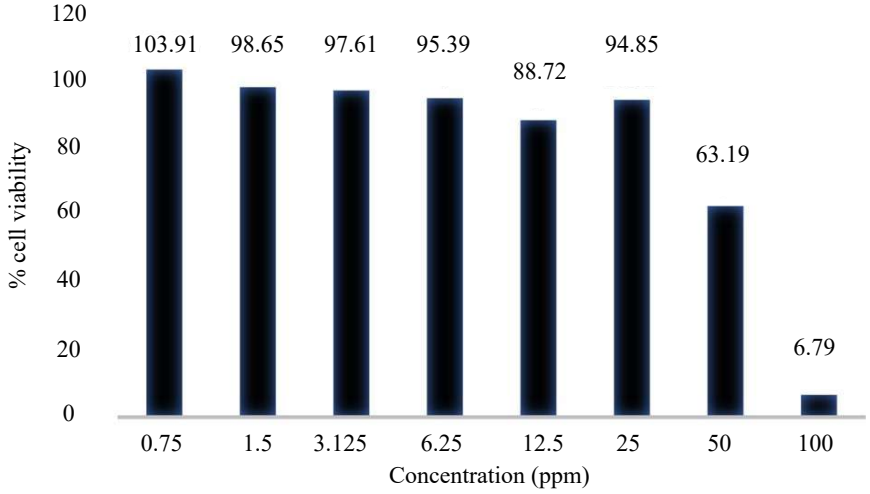

Figure 4. Viability percentage of mouse melanoma B16F10 cell line using the MTT assay treated with EAS

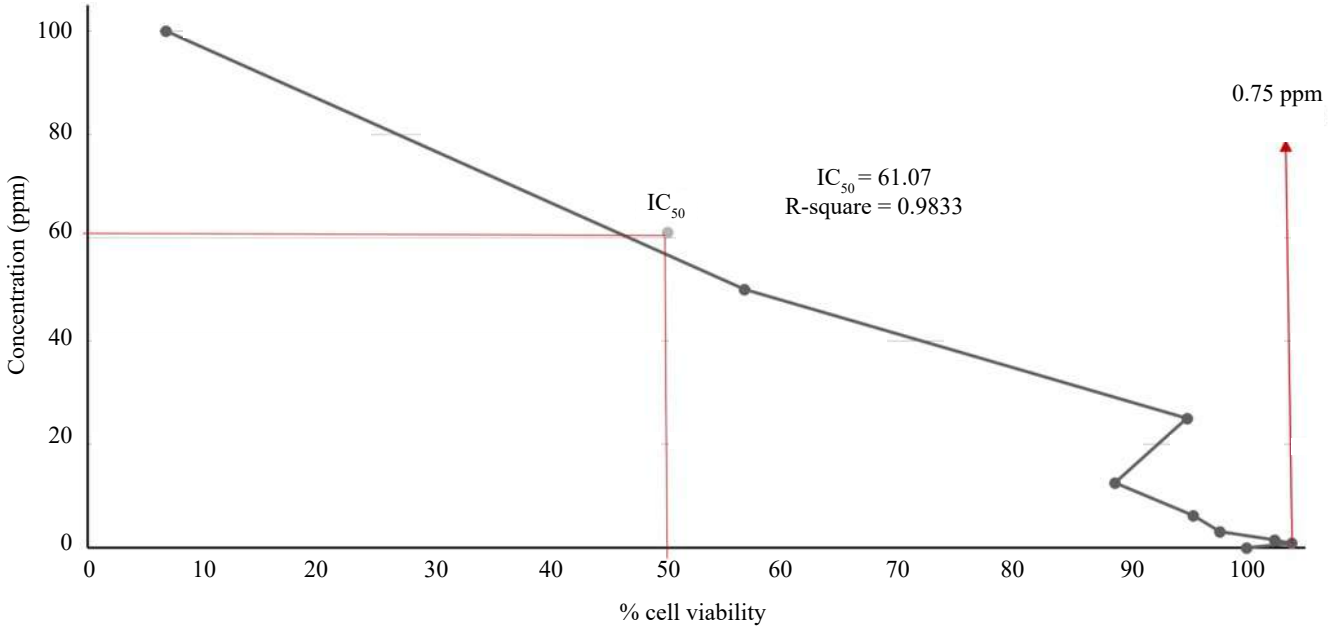

Figure 5. The IC_{50} value for the viability of EAS on the mouse melanoma B16F10 cell line

of 5% and substantially differed from concentrations above 3.125 ppm at a significance level of 10%.

Cell viability observations of the mouse melanoma B16F10 cell line after EAS administration at various concentrations were conducted using an inverted microscope camera (Dino-Lite) at 32x magnification, as depicted in Figure 6.

4. Discussion

Avocado fruit is generally consumed for its flesh, whereas the seeds are discarded and considered waste (Risyad *et al.* 2016). Avocado seeds account for 13-17% of the fruit and are rich in various functional and bioactive components, including polysaccharides, proteins, lipids, minerals, and vitamins (Bangar *et al.* 2022). The phytochemical contents of avocado seeds include flavonoids, tannins, saponins, and alkaloids (Angajala *et al.* 2019; Kopon *et al.* 2020; Setyawan *et al.* 2021) and triterpenoids (Angajala *et al.* 2019; Kopon *et al.* 2020), and phytosterols (Kopon *et al.* 2020). Among the natural phenolic compounds that act as tyrosinase inhibitors, flavonoids are considered to be the most effective (Panzella and Napolitano 2019). The *in silico* method from previous research demonstrated that catechin present in avocado seeds could function as an anti-tyrosinase agent, preventing melanogenesis and potentially serving as a skin-whitening agent (Laksmiani

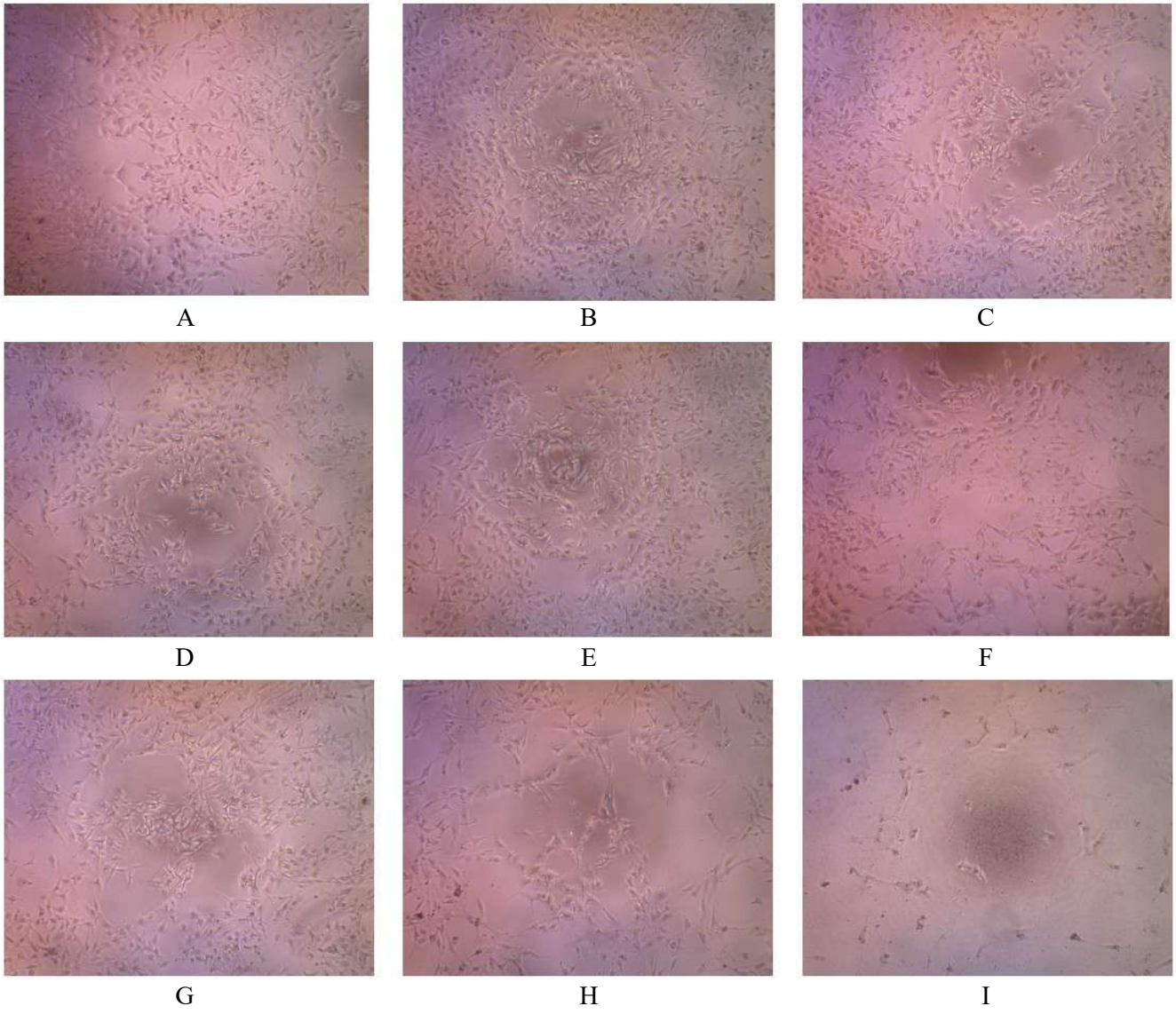

Figure 6. Morphology of mouse melanoma B16F10 cell line cells treated with EAS (magnification 32x). (A) Control, (B) 0.75 ppm, (C) 1.5 ppm, (D) 3.125 ppm, (E) 6.25 ppm, (F) 12.5 ppm, (G) 25 ppm, (H) 50 ppm, (I) 100 ppm

et al. 2020). Sato and Toriyama (2009) found that catechin inhibits melanin synthesis in B16F1 mouse melanoma cells. Another study by Widyastuti *et al.* (2023) discovered that catechin from gambir (*Uncaria gambir* (Hunter) Roxb.) reduced tyrosinase activity and melanin content in B16F0 cells. The phytochemical analysis results of EAS in this study align with those found in previous studies, indicating the presence of flavonoids (Asdar 2014; Anganjala *et al.* 2019), saponins (Anganjala *et al.* 2019), and steroids (Asdar 2014).

Antioxidants are compounds capable of neutralizing free radicals by donating electrons, thereby inhibiting chain reactions leading to free radical formation, which can induce oxidative stress or an imbalance between prooxidants and antioxidants (Alim *et al.* 2022). The antioxidant strength of the test compounds determined using the DPPH method can be categorized based on the IC_{50} values: powerful (IC_{50} <50 ppm), strong (IC_{50} 50-100 ppm), moderate (IC_{50} 101-150 ppm), and weak (IC_{50} >150 ppm) (Alim *et al.* 2022). The IC_{50} value of EAS is 89.47±0.73 ppm, indicating vigorous antioxidant activity. A study by Alim *et al.* (2022) revealed that avocado seed extract from Enrekang, Indonesia, exhibits powerful antioxidant potential with an IC_{50} value of 37.7475 ppm. Another study by Sutriningsih and Astuti (2017) reported an IC_{50} value of avocado seed extract as 15.39 ppm, categorizing it as a powerful antioxidant. In this study, the antioxidant activity was classified as strong. This difference may arise from various studies indicating that secondary metabolites, such as flavonoids, which act as antioxidants in plants, can vary in content across different regions. Such variations can be attributed to environmental factors, including light, temperature, pH, and altitude, which can influence the chemical composition of plants (Alim *et al.* 2022).

High-Performance Liquid Chromatography (HPLC) is used for separation analysis techniques including qualitative and quantitative analyses, separation/isolation, and purification. The fundamental principle of HPLC involves a dynamic adsorption process in which analyte molecules move through a porous gap. The column material (stationary phase) interacts with the sample components, leading to separation. The duration of the interaction, known as the retention time, is influenced by the strength of the interaction between the column material and sample components (Angraini and Desmaniar 2020). In a previous study using the Thin-Layer Chromatography Densitometry (TLC-Densitometry) method, the presence of catechin compounds at a concentration of 1 mg/ml was identified in the EAS (Laksmiani *et al.* 2020). Sanjaya *et al.* (2020) found a catechin content of 25.55% obtained from EAS, also utilizing the TLC-Densitometry method. The examination results of EAS in this study, using HPLC, further confirmed the presence of catechin, which is a secondary metabolite of avocado seeds.

Cell culture is an in vitro model of human melanoma (Overwijk and Restifo 2001). Mouse melanoma cells exhibit distinct characteristics based on their metastatic ability and mortality, namely, B16F0, B16F1, B16F10, and B16BL6 (Nakamura *et al.* 2002). The mouse melanoma B16F10 cell line possesses a melanogenic mechanism similar to human epidermal melanocytes, making it the most commonly used model for *in vitro* melanin synthesis assays (Kim and Hyun 2022). *In vitro* testing for tyrosinase inhibition using the mouse melanoma B16F10 cell line commenced with cytotoxicity assessments. The MTT assay is a simple colorimetric test (Kamiloglu *et al.* 2020) and the gold standard for determining cell viability and proliferation. MTT reagent is a mono-tetrazolium salt consisting of a positively charged quaternary tetrazole ring core containing four nitrogen atoms surrounded by three aromatic rings, including two phenyl groups and one thiazole ring (Ghasemi *et al.* 2021).

The addition of a tetrazolium salt disrupts the tetrazolium ring core (Ghasemi *et al.* 2021). In this assay, tetrazolium salt is reduced to formazan, a purple insoluble compound, by dehydrogenase present in the mitochondria of living cells at 37°C (Kamiloglu *et al.* 2020; Ghasemi *et al.* 2021). The formazan salt was dissolved by adding a solvent and the colored product was quantitatively measured at 570 nm using a multiple spectrophotometer (Kamiloglu *et al.* 2020). The formation of purple formazan indicates the presence of live cells, and the intensity of the purple color correlates with the number of viable cells (Bahuguna *et al.* 2017; Ma'arif *et al.* 2020; Widyastuti *et al.* 2023). Viable cells with active metabolism convert MTT into a purple formazan product with maximum absorption near 570 nm. The number of living cells is directly proportional to the intensity of the color in the culture (Kamiloglu *et al.* 2020).

The morphological characteristics of mouse melanoma B16 cell culture include mixed spindle-shaped and epithelial-like cells. Cell morphology in the culture after exposure to various concentrations of EAS showed concentration-dependent changes in cell morphology. Higher doses led to more significant changes in cell morphology, possibly because of cytotoxicity. Cytotoxicity in melanoma cells is marked by shrinkage and detachment from the tissue culture surface, reduced cell and nuclear volume, increased number of dead or

floating cells, cytoplasm condensation, and cell shrinkage, indicating cell death (Marvibaigi *et al.* 2016; Ndhundhuma and Abrahamse 2017). Visual observations in this study using an inverted microscope showed that the mouse melanoma B16F10 cell line remained adherent to the culture flask wall after exposure to EAS concentrations of 0.75-25 ppm. The cells did not exhibit signs of death, such as swelling or shrinking, and there was a purple formazan color, indicating that the cells were still alive. However, at 50-100 ppm concentrations, many cells died. The calculation of cell viability may become non-linear due to the biochemical mechanisms of cell death resulting from adenosine triphosphate (ATP) depletion and defects in the cell membrane. The suboptimal function of succinate dehydrogenase, an enzyme responsible for ATP production, can lead to its depletion. Enzymes that are inactive or less effective in ATP generation hinder cell functional activity, ultimately reducing cellular performance and leading to cell death. Defects in the cell membrane can affect mitochondrial activity, which is crucial for ATP production, causing a reduction in ATP levels and subsequent cell death. This damage renders the cell non-viable, ultimately leading to cell death (Ma'arif *et al.* 2020).

Research on the effects of EAS on melanin synthesis and its cytotoxicity in the mouse melanoma B16F10 cell line is yet to be reported. A cytotoxicity assay using the MTT method was previously conducted by Hindritiani *et al.* (2013), who noted that the methanol extract of the malaka fruit (*Phyllantus emblica*) had a cell viability of over 90% in mouse melanoma B16. Another study on the extract of Ranunculus chinensis Bunge, measured using MTT on the mouse melanoma B16F10 cell line, revealed that cell viability reached 100% (Kim *et al.* 2022). In their study, Widyastuti *et al.* (2023) indicated that 90% and 98% of catechins isolated from *U. gambir* had cell viabilities of approximately 60% and 70%, respectively, against B16F0 melanoma cells. We argue that the cytotoxicity of plant extracts is highly dependent on the type of extract and cells used. Different extracts contain different compounds, and other cell types exhibit varying resistance. In this study, the viability of EAS, ranging from 80-100%, indicated that EAS is relatively non-cytotoxic. Therefore, in this study, we explored EAS as a tyrosinase inhibitor in the mouse B16F10 melanoma cell line, potentially functioning as a natural whitening agent.

Conflict of Interest

The authors declare no conflicts of interest.

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