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RESEARCH ARTICLE

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The µDrop Method Enhances Melanin Content Measurement in the *in vitro* Melanogenesis Model Using B16F10 Cell Line

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Background: The B16F10 cell line is a cell frequently used in melanin content assays. However, reports on cell models using B16F10 are limited, particularly as the robust model cell in the Indonesian cosmetics industry. We found measuring melanin content using microplate spectrophotometry to be challenging, so this research was conducted to develop a method using µDrop spectrophotometry.

Materials and methods: In this *in vitro* study, the B16F10 melanoma cell line was cultured in Roswell Park Memorial Institute (RPMI) medium containing 5% fetal bovine serum (FBS). The cells were categorized into control, stimulated, and treated groups. Melanogenesis stimulation was achieved using 1µM α-melanocyte-stimulating hormone (α-MSH), while inhibition using 800 µg/ml kojic acid. After treatment, the cells were incubated for 48 hours. Their melanin content was then measured using an ELISA reader with a µDrop method and compared with the microplate method. Statistical analysis used a one-way ANOVA test with Turkey's Post Hoc analysis.

Results: The µDrop method increased the melanin signal into the linear range of machine readings, while the signals from the microplate method fell far below this range. The B16F10 melanoma cell lines stimulated by α-MSH exhibited increased melanin production compared with the control group, while kojic acid treatment significantly reduced (p<0.05) melanin content in the stimulated group.

Conclusion: The µDrop method significantly outperformed the microplate method in measuring melanin content within melanogenesis cell models, offering enhanced accuracy and particularly excelling at quantifying low content of melanin.

Keywords: *µDrop, microplate, melanin, melanogenesis, B16F10 cell line, RPMI*

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Introduction

Melanogenesis, the process through which melanocyte cells produce melanin pigment, is influenced by various internal and external factors, including exposure to ultraviolet (UV) light.[1] Upon UV radiation absorption by the skin, the tyrosinase enzyme is activated, and melanin formation is initiated. Melanin mitigates UV radiation's harmful effects on cutaneous DNA cells.[2] Nonetheless, aberrant melanin and tyrosinase accumulation in cells can result in skin conditions such as melasma and hyperpigmentation.[3,4] Although melasma and skin hyperpigmentation is not inherently dangerous, it can substantially diminish the quality of life for specific individuals. A prevalent approach to addressing hyperpigmentation involves skin lightening through melanin synthesis inhibition, one of which is kojic acid.[3]

Kojic acid is a common compound for skin lightening, functioning by retarding melanin biosynthesis and impeding the tyrosinase enzyme activity.[5,6] Kojic acid is produced through the aerobic fermentation process of certain fungi, including *Aspergillus* and *Penicillium*. It is a fundamental component in developing novel tyrosinase inhibitors.[3,5,6] As a hydroxypyranone derivative, it hinders tyrosinase activity through copper chelation and binding to the enzyme's active site.[5,6] Copper ions catalyze tyrosinase activity upon exposure to UV light, but kojic acid combats this process by chelating copper ions, inhibiting tyrosinase activation and melanin production.[3] Kojic acid is a widely utilized cosmetic ingredient that has been extensively studied for its efficacy in skin whitening. In addition to kojic acid, hydroquinone and arbutin are frequently employed for skin-lightening purposes. However, hydroquinone is associated with adverse effects that can result in permanent skin lightening. Hence, kojic acid is often favored as a safer alternative.[6] Meanwhile, when compared to arbutin, kojic acid shows a more consistent melanogenesis inhibitory effect than arbutin as a positive control.[7]

Melanocyte cells are capable of undergoing transformation and rapid proliferation, which can lead to the development of melanoma.[3,8,9] Melanin expression in melanoma cells is a crucial indicator for understanding the nature of melanoma and its responsiveness to various therapeutic interventions.[10] The B16 mouse melanoma cell line is widely employed as a cultured cell model and is particularly effective in assessing melanogenesis inhibition *in vitro*.[3,11] This cell line includes a derivative, B16F10, which is a critical 2D culture cell model for assessing substances that impact melanin production and release. This is essential to remember that when B16 cells are cultured in a 2D environment, they may progressively lose their ability to produce melanin. This loss of capacity necessitates prior stimulation for depigmentation studies.[12] A stimulant, α-melanocyte-stimulating hormone (α-MSH) is a commonly used stimulant for these studies, as it is well-known for activating the signaling pathway involved in melanin production. The main signaling pathway for melanogenesis is the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway as the main pathway for melanogenesis.[12,13]

Melanin content in cultured cells can be analyzed by five methods, including melanin visualization in cell pellets, melanin visualization by light microscopy, melanin quantification by spectrophotometry, melanin quantification by other spectroscopy methods, and high-performance liquid chromatography.[11] Spectrophotometry is the most widely used method because the research procedure is simple and does not require extensive equipment or skilled operators.[11,12] Many studies use 100 µL volume spectrophotometry reading, but there have been no reports on using the lower volume, significantly below 10 µL, such as using the µDrop plate.

The B16F10 melanoma cell line is a crucial and robust model for the cosmetics industry in Indonesia; however, reports on its use in cell models are still relatively scarce. Therefore, this research was conducted to address this gap by developing a cell culture model specifically for evaluating melanogenesis. The study evaluated the effectiveness of a-MSH in stimulating melanin synthesis and the inhibitory impact of kojic acid, a commonly used depigmentation substance. This is crucial to confirm that the B16F10 cell line can produce melanin when cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% fetal bovine serum (FBS). This capability is necessary for conducting experiments on inhibiting melanin synthesis involving other substances.

Materials and methods

Cell Culture Preparation

The research used an *in vitro* model that involved the B16F10 cell line at the Health, Safety, and Environment (HSE) Laboratory, Universitas Padjadjaran. The B16F10 cell line, obtained from the HSE Laboratory, was thawed,

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and then cultured in RPMI containing 5% FBS and 1% Penicillin/Streptomycin. The RPMI (PAN-Biotech, Aidenbach, Germany), FBS (PAN-Biotech), and Penicillin/Streptomycin (PAN-Biotech). After that, the cells were placed in a cell culture incubator (37°C and 5% CO_2).

Cell Treatment Preparation

The cells were grown in culture and distributed into two treatment groups and a control group. The control group consisted of cells that were not treated with anything, while the treatment groups were divided into the stimulated group and the treated group. Cells in the stimulated group were treated with α-MSH, whereas cells in the treated group were stimulated with α-MSH and then treated with kojic acid. Alpha-melanocyte-stimulating hormone (α-MSH) (Cat. No. M4135, Sigma-Aldrich, St. Louis, Missouri, US) was used to induce melanin synthesis. Kojic acid (Aogubio, Xi'an, China) was used to inhibit melanin synthesis.

Melanin Content Test

The cells were initially distributed in 24-well plates at a density of $5x10^4$ cells/well and cultured in 1 mL of a complete RPMI medium. Subsequently, they were placed in a cell culture incubator. Following a 24-hour incubation period, the medium was aspirated, and 1 mL of medium containing α-MSH with or without kojic acid (at a final concentration of 1 μM α-MSH and 800 μg/mL kojic acid) was introduced into each well. After a 48-hour treatment duration, the cells were harvested using 0.25% trypsin (Servicebio, Wuhan, China) with culture medium, followed by centrifugation at 12,000 rpm for 10 minutes. The resulting cell pellet was added with 30 μL of 1 N NaOH for measurements using a μDrop plate (Thermo Fisher Scientific, Waltham, MA, US) and 100 μL of 1 N NaOH for measurements using a microplate (NEST, Wuxi, China). The samples were then incubated at 95°C for 15 minutes in a dry bath. All experiments were performed in triplicate, as adjusted from the previously reported method.[14,15] Melanin content determination was quantified at a wavelength of 405 nm with an ELISA reader and microscopic imaging analysis.[16] Subsequently, the relative melanin content was calculated using the following formula:

[Absorbance of sample / Absorbance of control] x 100%.[15]

Cell Viability Test

Cell viability percentage was measured by employing the WST-1 assay to determine the non-toxicity of kojic acid on the cells. Specifically, cells were plated at a density of $2 x10^4$ cells/well in 96-well plates and cultured in 100 μL of complete RPMI medium. After a 24-hour incubation period, the medium was aspirated, and 1 μL of medium containing α-MSH with or without kojic acid (final concentration: 1 μM α-MSH and 800 μg/mL kojic acid) was added to each well. Following a 48-hour treatment period, the medium was aspirated, and 90 μL of RPMI medium without FBS and 10 μL of WST-1 reagent (Sigma-Aldrich, St. Louis, Missouri, US) were added to all wells. Subsequently, the cells were incubated for 4 hours at 37°C in an atmosphere containing 5% CO_2. After incubation, the cells were homogenized for 1 minute using a shaker. Absorbance readings were obtained at a wavelength of 475 nm using an ELISA reader, with three replicates performed. The cell viability percentage was computed with the following formula:

[Absorbance of sample / Absorbance of control] x 100%.[15]

Results

In this study, the control group (Figure 1A), the cells exhibited light pigmentation, indicating basal melanin production without any external stimulation. In contrast, the stimulated group, the cells treated with 1 μM α-MSH, displayed visibly darker pigmentation, reflecting a substantial increase in melanin synthesis. When these stimulated cells were subsequently treated with kojic acid, a marked reduction in pigmentation was observed. In the control group, the cells displayed minimal melanin synthesis with weak dendritic pigmentation. In the stimulated group (Figure 1B), a marked increase in melanin production was evident, particularly at the dendritic ends of the cells, which appeared heavily pigmented. However, in the treated group (Figure 1C), kojic acid administration led to a notable reduction in melanin content, with markedly less pigmentation observed under the microscope.

The quantitative analysis of melanin content supported the microscopic findings (Table 1). The α-MSH-stimulated cells showed an increase in melanin content when using a μDrop plate. Upon treatment with kojic acid, the melanin content decreased. Similar trends were observed when using a microplate, though the absolute values were lower.

The melanin content in control group was 100±6.69%, while stimulated group exhibited 128.90±29.26%, and treated group showed a decrease to 59.50±17.00% (Figure 2). There was a significant difference among the control, stimulated, and treated groups (One-way

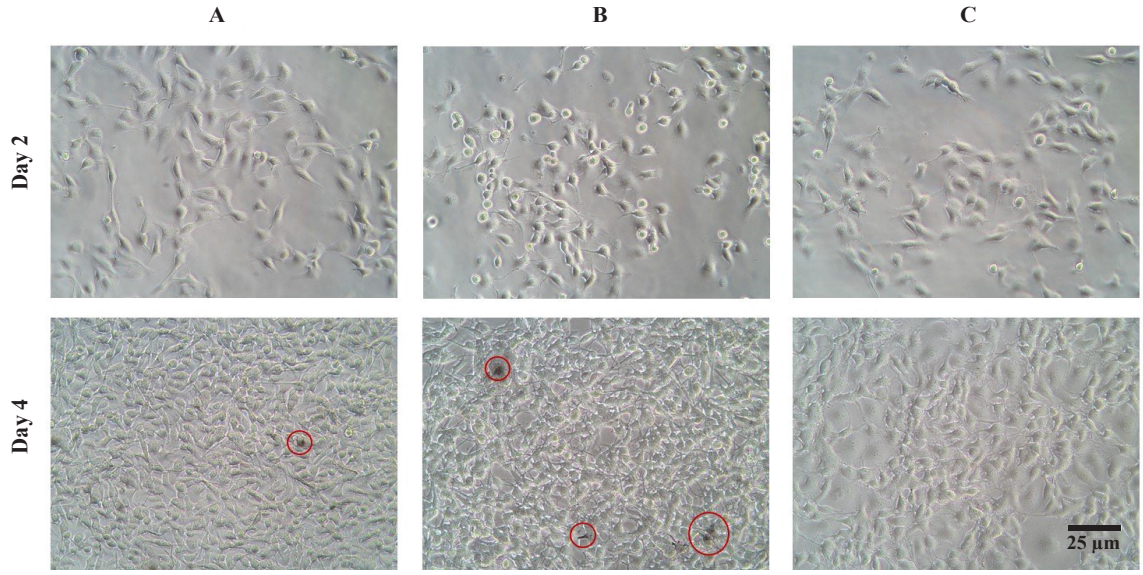

Figure 1. The increase of melanin production in B16F10 cells occurred after 48 hours of stimulation, while treated cells showed no melanin production. Day 2 represented the day following a 24-hour incubation period and preceded the addition of α-MSH and kojic acid. Day 4 represented the period after 48 hours of treatment with α-MSH and kojic acid. B16F10 cells produced melanin, shown with the red circles. A: Control group. B: Stimulated group. C: Treated group. Black bar: 25 μm.

ANOVA, p=0.0149). The melanin content in treated group (69.4±16.26%) was significantly different (Tukey's post hoc test, p=0.0125) than the ones in the stimulated group.

The cell viability percentage in control group was 100±0.00%, while stimulated group exhibited 95.43±4.80%, and the treated group showed 98.18±3.16% (Figure 3). Treated group did not achieve 100% viability but remained higher than the ones in stimulated group.

Discussion

This study demonstrated that the B16F10 cell line, when stimulated with 1 μM α-MSH, can enhance melanin production, as evidenced by microscopic imaging. However, as in many protocols, the melanin content measurement by spectrophotometry at a 100 μL volume, as stated in some protocols, did not show values in the linear range

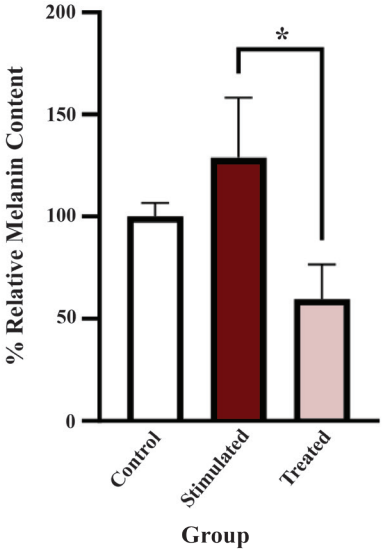

Figure 2. Kojic acid reduced the percentage of melanin content in B16F10 cells. Bars shows a significant decrease in melanin content based on Tukey's post hoc test results with p<0.05. The data were presented as means±SD with a sample size of n=3.

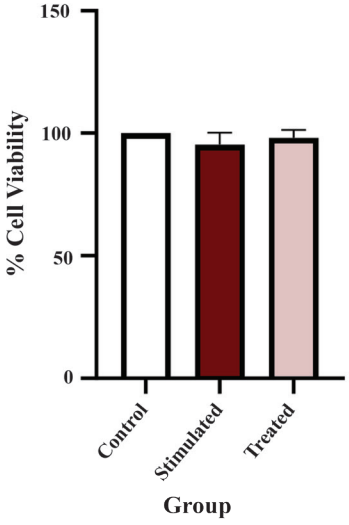

Figure 3. Kojic acid maintained high cell viability percentage in B16F10 cells. Bars shows percentage of viable cells. The data were presented as means±SD with a sample size of n = 3.

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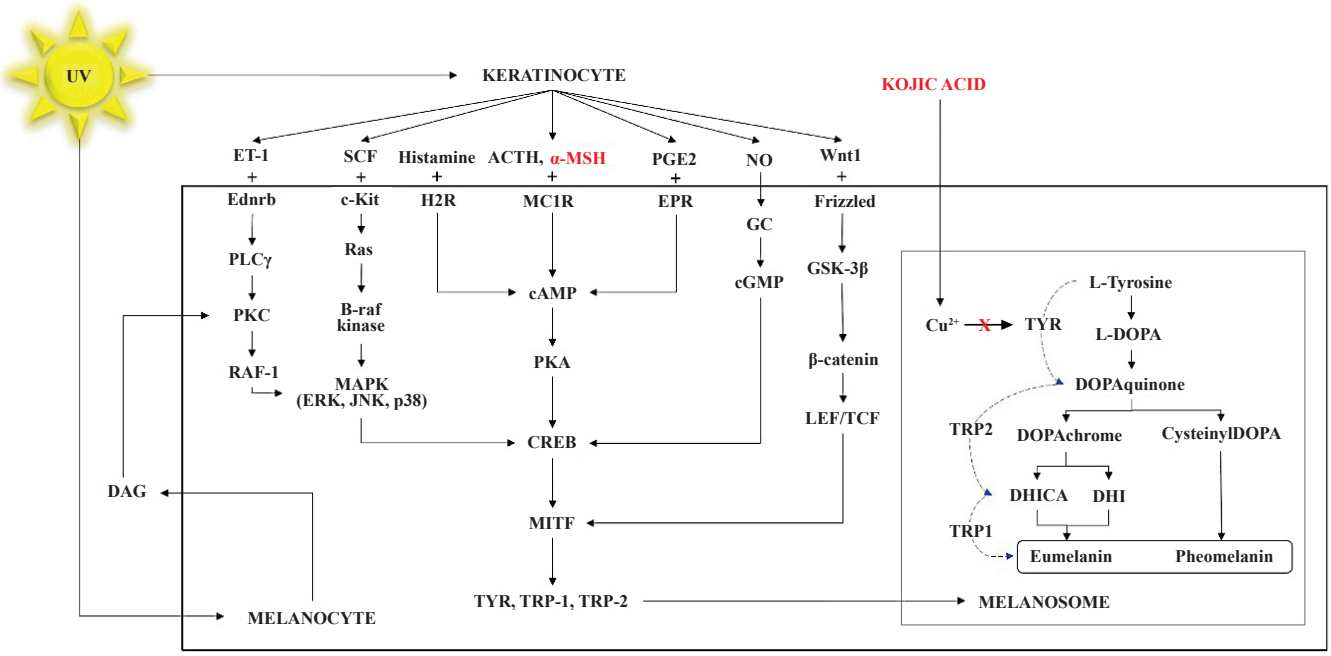

Figure 4. Melanogenesis is triggered by α-MSH and suppressed by kojic acid.

of the machine reading.[12,16] Hence, there was no statistical significance (Table 1). In the μDrop method, the melanin from the cell was first dissolved in only 30 μL NaOH, and then 2 μL was taken for reading using a μDrop plate. After developed and switched the measurement to the μDrop plate method, the melanin readout values fell in the linear range of the machine reading. Therefore, the results followed the microscopic results (Figure 1) and reached statistical significance (Table 1 and Figure 2).

Low absorbance values when using microplates may be caused by a low cell count or melanin production, resulting in low absorbance after NaOH treatment. Therefore, this study established the B16F10 cell line as an effective model for melanogenesis assessment and introduced an optimized method for evaluating melanin content in low melanin production. The utilization of μDrop plates in prior studies has yet to be documented. Melanin content measurements with a μDrop plate are applied when cells or melanin are produced in low quantities. The medium type and concentration of supplementation may account for the low melanin production observed in this study. The product documentation specifies using Dulbecco's modified eagle's medium (DMEM) containing 10% FBS as the ideal medium for the B16F10 cell line. Still, this study used RPMI containing 5% FBS. In a study, amelanotic B16F10 cell lines cultured using DMEM showed a significant increase in melanin content compared to cells cultured with RPMI. This is because DMEM contains more L-tyrosine than RPMI. L-tyrosine is an amino acid needed in the melanin synthesis process and can also be used as an agent to induce melanogenesis.[17] Nevertheless, this study's application of RPMI supplemented with 5% FBS can still stimulate melanin production in the B16F10 cell line for melanogenesis assays. This outcome concurs with another investigation, which used an RPMI medium containing 10% FBS to evaluate melanin content.[16]

In addition to the choice of medium, the low production of melanin significantly influences the amount of NaOH administered during the final stage of the melanin content test. This study revealed that administering 100 μL of NaOH, as in previous studies, resulted in a low absorbance value that was uniformly observed across all experimental groups. Furthermore, variations in the final volumes following high-temperature incubation have been shown to impact the absorbance value of each sample, potentially yielding divergent absorbance values within a single experimental group. To solve this, NaOH was added after a 15-minute incubation at 95°C to standardize the final volume of each sample, which was subsequently quantified using spectrophotometry via μDrop plates. The comparison of the volume of NaOH administered to the cell pellet and the final volume after high-temperature incubation may significantly influence the resultant concentration of the sample, consequently affecting the absorbance value.

Table 1. Melanin content in B16F10 cell line was higher using μDrop method than microplate method.

Group	Melanin Content	
	μDrop plate	Microplate
Control group	0.165±0.011	0.056±0.004
Stimulated group	0.212±0.048	0.063±0.003
Treated group	0.098±0.028	0.057±0.001

This observation is consistent with the Beer-Lambert Law, which stipulates a linear relationship between the sample concentration and the absorbance value. Accordingly, lower sample concentrations yield reduced absorbance values and vice versa.[18]

The B16F10 cell line can enhance melanin production when stimulated with 1 μM α-MSH, as evidenced by microscopic imaging and the measurement of melanin content using spectrophotometry with μDrop plates. These findings were in line with previous research, which observed an increase in melanin synthesis in the B16F1 and B16F10 cell lines following exposure to α-MSH within a range of 0.001-10 nM.[12] In another study, α-MSH with a dose of 0.1-0.3 μM increased melanogenesis influenced by the size of the dose.[19] In the same study that using 1 μM α-MSH was significantly elevated melanin synthesis compared to the cell without α-MSH.[20] Melanin synthesis occurs within melanocyte cells in the melanosome, which then migrates from the dendritic end to the keratinocytes.[9,21] This intricate process, known as melanogenesis, begins with the secretion of α-MSH by keratinocytes, subsequently binding to the melanocortin-1 receptor (MC1R).[2] The resultant α-MSH-MC1R complex activates a signaling pathway that regulates the microphthalmia-associated transcription factor (MITF) and the downstream gene expression.[21] The mechanism is illustrated in Figure 4.

The α-MSH is a commonly utilized compound for stimulating melanin production in various melanogenesis assays. The production is triggered by exposure to UV radiation. The UV radiation activates the melanogenesis pathway by producing specific factors by keratinocytes, which can induce or inhibit melanin production. Internal factors that induce melanogenesis include nitric oxide (NO), stem cell factors (SCF), adrenocorticotropic hormone (ACTH), endothelin-1 (ET-1), prostaglandin E2 (PGE2), and histamine. Subsequently, these factors activate various melanogenesis pathways, such as cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), nitric oxide (NO)/cyclic guanosine monophosphate (cGMP), SCF/c-Kit, diacylglycerol (DAG)/protein kinase C (PKC), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and wingless-related integration site (Wnt)/β-catenin pathway.[22]

The α-MSH, as one of the paracrine factors, acts as a significant molecule that binds to its receptor and triggers the activation of adenylate cyclase, leading to an increase in cAMP levels within melanocytes. Subsequently, the activated cAMP stimulates PKA. The PKA is activated, phosphorylating and activating the transcription factor cAMP response element binding protein (CREB). The activated CREB binds to the m-MITF promotor, activating MITF transcription and initiating MITF transcription. This sequence depicts the signaling cascade of the cAMP/PKA pathway.[21] Comparatively, the induction of melanogenesis through α-MSH exhibits higher melanin production than that induced by UVA or UVB radiation.[19]

Melanogenesis is facilitated by the transcription factor MITF, which governs the expression of several target genes associated with melanin production, including tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1), and dopachrome tautomerase or tyrosinase-related protein-2 (TRP-2).[21] These enzymes, particularly tyrosinase, are pivotal in regulating melanin synthesis in melanocytes.[1,9] Tyrosinase serves as a precursor enzyme, orchestrating the hydroxylation of L-tyrosine into L-DOPA and subsequently catalyzing the oxidation of L-DOPA into DOPAquinone,

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driving melanin production.[1,9,23] DOPAquinone then converted become 5,6-dihydroxyndole-2-carboxylic acid (DHICA) by TRP-2 and DHICA oxydized become eumelanin by TRP-1 in neutral pH.[21] The tyrosinase activity is notably influenced by copper ions at its active site.[3]

This study demonstrated that kojic acid can inhibit the formation of melanin in the B16F10 cell line stimulated with 1 μM α-MSH to prove that the cells used can not only stimulate but also inhibit melanin production. This results were in line with the previous research which showed that 500 μg/ml of Kojic acid significantly lowered melanin content compared to controls.[20] In another study also showed that kojic acid at a concentration of 7.5-120 μM inhibited the activity of tyrosinase enzyme and decreased melanin content in melanoma B16F10 cells line.[7] Moreover, prior studies confirmed the non-cytotoxic nature of kojic acid at concentrations of 125-500 μg/mL in B16F10 cells.[20] Notably, the current study endorses the tolerability of an 800 μg/mL concentration of kojic acid on the B16F10 cell line (Figure 3). This proves that the decrease in melanin content occurs not due to cell death. Kojic acid demonstrates the capacity to inhibit tyrosinase activity through copper chelation and binding to its active site. This property allows kojic acid to impede tyrosinase activation for melanin synthesis.[3,5,6] The process of melanogenesis is a consequence of skin exposure to UV rays and the ensuing oxidative stress. Consequently, antioxidants can serve as an effective component in inhibiting the melanogenesis process. In a previous study was demonstrated that the use of polyphenols such as protocatechuic acid and ferulic acid as antioxidants effectively inhibits the activity of tyrosinase enzymes. Antioxidants can neutralize free radicals and chelate metals, thus shielding the skin from radiation-induced damage, which could otherwise trigger overexpression of the tyrosinase gene, leading to hyperpigmentation.[24] Additionally, antioxidants can impede the activation of the MAPK/ERK signaling pathway, a key pathway in the process of melanogenesis, by reducing UV-induced reactive oxygen species (ROS), as highlighted in another study. The administration of probiotic supplementation was shown to enhance the production of enzymatic antioxidants, thereby reinstating the equilibrium between antioxidants and free radicals in the skin and preventing adverse effects resulting from exposure to UVB rays.[25]

The study was limited because this study utilized only a single compound, To improve the findings of this study, further study should investigate the effectiveness of various compounds in either inducing or inhibiting melanogenesis, which would enable a comparative analysis. Furthermore, examining melanin production in both RPMI medium and DMEM would be advantageous, along with studying the effects of varying concentrations of FBS, specifically 5% versus 10% supplementation.

Conclusion

A novel modification was made when microplate analysis was impractical due to very low melanin concentrations. This modification employed a spectrophotometry protocol where using the μDrop plate with 2 μL volume enabled accurate melanin quantification. The μDrop plate presents a superior option over microplates for melanin content assays due to efficiency in sample volume and reduced measurement interference. These findings highlight the μDrop plate method as the superior choice for researchers seeking reliable and precise measurement in studies related to melanin production.

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Authors' Contributions

SE, GIN, and DY conceived and planned the research. DY and MMK performed data acquisition and collection. SE, GIN and DY processed the experimental data and conducted the analysis. LF wrote the manuscript and created the visual aids. MHB assisted in interpreting the results. SE, GIN, DY, MMK, LF, and MHB contributed to the critical revision of the manuscript.

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