

Research Article

Depigmenting Effect of Kojic Acid Esters in Hyperpigmented B16F1 Melanoma Cells

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The depigmenting effect of kojic acid esters synthesized by the esterification of kojic acid using *Rhizomucor miehei* immobilized lipase was investigated in B16F1 melanoma cells. The depigmenting effect of kojic acid and kojic acid esters was evaluated by the inhibitory effect of melanin formation and tyrosinase activity on alpha-stimulating hormone- (α -MSH-) induced melanin synthesis in B16F1 melanoma cells. The cellular tyrosinase inhibitory effect of kojic acid monooleate, kojic acid monolaurate, and kojic acid monopalmitate was found similar to kojic acid at nontoxic doses ranging from 1.95 to 62.5 $\mu\text{g}/\text{mL}$. However, kojic acid monopalmitate gave slightly higher inhibition to melanin formation compared to other inhibitors at doses ranging from 15.63 to 62.5 $\mu\text{g}/\text{mL}$. Kojic acid and kojic acid esters also show antioxidant activity that will enhance the depigmenting effect. The cytotoxicity of kojic acid esters in B16F1 melanoma cells was significantly lower than kojic acid at high doses, ranging from 125 and 500 $\mu\text{g}/\text{mL}$. Since kojic acid esters have lower cytotoxic effect than kojic acid, it is suggested that kojic acid esters can be used as alternatives for a safe skin whitening agent and potential depigmenting agents to treat hyperpigmentation.

1. Introduction

Melanin is synthesized via melanogenesis process to give pigment of skin, brain, eye, and hair [1–3]. Tyrosinase is a key enzyme that is responsible for melanogenesis in melanoma and melanocytes [4, 5]. The inhibition of tyrosinase will greatly affect the melanogenesis process and melanin production. The occurrence of abnormal melanin production is the cause for many hyperpigmentation, postinflammatory pigmentation, melasma, and skin-aging process [6–8]. Kojic acid is a well-known antityrosinase agent, efficiently used for skin lightening cosmetic products and widely used to treat hyperpigmentation, melasma, and wrinkle [5, 9–11]. However, most of the kojic acid and its derivatives are not oil soluble and unstable at high temperature for long term storage, prohibiting them to be directly incorporated in oil base cosmetic and skin-care products. Therefore, a few attempts

had been made to improve the physical properties and biological activities of kojic acid (KA) via esterification with fatty acids aimed at better industrial application [12–14].

The physical properties of kojic acid esters (KA esters) are important factor for inhibition of melanin synthesis where it must penetrate into the cell membrane to inhibit cellular tyrosinase and melanin synthesis. Thus, appropriate hydrophobic and hydrophilic balance of the derivatives is important for the inhibition of melanin synthesis [15]. The improvements of the characteristics of depigmenting agents are very important to enhance their applications in cosmetic and skin-health industries. Reports on the antimelanin and tyrosinase inhibitory of KA esters in cell system are not available in the literature.

The objective of this study was to analyze the cytotoxicity and depigmenting activities of KA and KA esters such as KA monooleate (KAMO), KA monolaurate (KAML), and

KA monopalmitate (KAMP) in B16F1 melanoma cells. KA was produced by the fermentation employing *Aspergillus flavus* link and KA esters were produced by the esterification of purified KA with various fatty acids using immobilized lipase.

2. Materials and Methods

2.1. Materials. Immobilized lipase from *Rhizomucor miehei* (RMIM) was purchased from Novo-Nordisk (Denmark). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin, and streptomycin were purchased from Invitrogen (Grand Island, NY, USA). Glycerol tributyrates, L-dihydroxyphenylalanine (L-DOPA), L-tyrosine, mushroom tyrosinase, phenylmethanesulfonyl fluoride (PMSF), L-ascorbic acid, and alpha-melanocyte stimulating hormone (α -MSH) were purchased from Sigma-Aldrich (Steheim, UK). All other chemicals and solvents used in this study were the analytical grade.

2.2. Production of Kojic Acid. KA was produced through the fermentation by *Aspergillus flavus* link 44-1 according to the method as described by Mohamad and Ariff [16]. In this method, the fermentation medium consisted of glucose (100 g/L), yeast extract (5 g/L), potassium dihydrogen phosphate (1 g/L), magnesium sulphate (0.5 g/L), and methanol (10 mL/L). The fungal spores suspension (1×10^5 spores/mL) was inoculated into 100 mL medium in 250 mL shake-flasks. The flasks were incubated at 30°C and agitated at 250 rpm for 5 days. The mycelia were filtered, washed, and transferred into 200 mL medium containing 100 g/L glucose in 500 mL shake flasks. The flasks were incubated in an orbital shaker at 30°C and agitated at 250 rpm for 30 days for the conversion of glucose into KA by the actions of cell-bound enzymes in nongrowing mycelia.

Cell mycelia were separated from the broth by centrifugation at 10 000 g (GRX-250, Tomy Seiko Co., Ltd. Japan). The supernatant containing KA was concentrated to get a final KA concentration of above 80 g/L using rotary evaporator (BUCHI model R-220, Germany). The concentrated KA solution was kept at 30°C for 24 h for crystallization. The KA crystals were separated by centrifugation and redissolved in methanol for the subsequent crystallization process to remove further pigments and impurities. High-purity KA (99.8%) was obtained after recrystallization with methanol for three times.

2.3. Enzymatic Synthesis of Kojic Acid Esters. The esterification of KA to KA esters (KAMO, KAML, and KAMP) was performed according to the method previously described [12, 17]. The reaction mixture for esterification process consisted of fatty acids (oleic acid, lauric acid, and palmitic acid), KA, and immobilized lipase in acetonitrile. The flasks containing the reaction mixture were incubated in a horizontal water bath shaker at 50°C, agitated at 180 rpm for 42 h. The reaction was terminated, by removing the enzyme from the mixture through filtration using filter paper. KA esters were

purified using crystallization method similar to that used for KA.

KA esters were examined by thin layer chromatography (TLC) on precoated silica gel plate (60F₂₅₄) and developed in hexane/ethyl acetate (70 : 30, v/v). The developed bands were visualized using UV light. Then, the products were analyzed on Agilent gas chromatograph after being silylated to TMS derivatives using a nonpolar column ZB-5HT Inferno (15 m \times 0.53 mm \times 0.15 μ m) with nitrogen as carrier gas. The oven temperature was programmed to rise from 100°C to 225°C at 15°C min⁻¹, and to 280°C at 30°C min⁻¹ for 1 min. The injector and flame ionization detectors were set at 340°C, and 350°C respectively. The composition of product was quantified by an integrator with 1,2,3-tributyrylglycerol as internal standard.

2.4. Characterization of KA Esters. The GC-mass spectrometry (GC-MS) analysis of the product isolated using preparative column chromatography was performed on a Perkin Elmer Instrument model Clarus 600 MS spectrometer. The GC was equipped with a non-polar column, ZB-5HT (30 m \times 0.32 mm \times 0.25 μ m). The carrier gas was helium at a flow rate of 1.5 mL min⁻¹. Proton NMR (1H-NMR) and carbon NMR (13C-NMR) spectra were obtained using Varian NMR Unity Inova 500 MHz with Pulsed Field Gradient. The samples were dissolved in deuterated chloroform with tetramethylsilane as internal standard. On the other hand, FTIR spectra were recorded on a Perkin Elmer 100-series FTIR spectrophotometer using Universal Attenuated Total Reflectance (UATR). The IR spectra were used to identify the possible molecular structures for the pure components and also used to determine the chemical changes during the reaction.

2.5. Cell Culture. B16F1 melanoma cells were purchased from American Type Culture Collection (ATCC). The cells were cultured in DMEM with 10% w/v fetal bovine serum and 1% w/v penicillin/streptomycin (100 IU/50 μ g/mL) in humidified atmosphere containing 5% CO₂ in air at 37°C. B16 cells were cultured in 96-well plates and 24-wells plates for different assays. All the experiments were repeated at least in triplicates.

2.6. Determination of Cell Viability. Cell viability was assessed by the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay with a slight modification [18]. B16F1 melanoma cells (1×10^5 cells/well) were seeded in a 96-well microtiter plate and allowed to adhere completely to the plate overnight [19]. On the next day, the medium was removed and a new medium containing test compounds with doses ranging from 1.9 to 500 μ g/mL was added to the plate and then incubated at 37°C in CO₂ incubator. After a total of 72 h incubation, medium was removed and 50 μ L of MTT solutions (1.0 mg/mL) was added to each well and the incubation was continued for 4 h. Then, formazon was solubilized in dimethyl sulfoxide (DMSO) and the absorbance was measured at 450 nm (reference at 630 nm)

using MR-96A microplate reader. DMSO at a toxic concentration of 5% v/v was used as a negative control [20].

2.7. Determination of Melanin Content. The release of extracellular melanin was measured according to the method described elsewhere [21]. In brief, B16F1 melanoma cells were seeded into 24-wells tissue culture plates at 1×10^4 cells/mL and incubated for 24 h. Then, α -MSH (0.1 μ M) was added and cells were treated with increasing doses of KA and KA esters for a total of 72 h incubation. After washing twice with phosphate buffered saline, cells were dissolved in 1 mL of 1 N NaOH. For measurement of melanin content, 100 μ L aliquots of solution were then placed in 96-well plates and the absorbance was measured at 450 nm using microplate reader. Antimelanin activity was expressed by the percentage of melanin content in KA and KA esters to that of untreated melanoma cells. L-ascorbic acid, a widely used whitening agent, was used as a reference [22].

2.8. Determination of Cellular Tyrosinase Activity. Cellular tyrosinase activity was determined according to the method described by Shin et al. [23]. In this method, B16F1 melanoma cells were treated with α -MSH alone and α -MSH with the addition of KA and KA esters at various doses for a total of 72 h incubation. The cells were washed with ice-cold PBS then lysed with 100 μ L phosphate buffer (pH 6.8) containing 1% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Then, lysate was clarified by centrifugation at 800 rpm for 5 min. The supernatant (100 μ L) was added into 50 μ L of L-DOPA (1 mM) and 50 μ L of L-Tyrosine (2 mM); and the mixtures were placed in a 96-well plate. During the incubation at 37°C, the absorbance was read at 492 nm at every 30 min for 3 h using a microplate reader. L-ascorbic acid was used as a reference.

2.9. Determination of Mushroom Tyrosinase Activity. KA and KA esters at various doses were dissolved in DMSO, 50 μ L of L-DOPA (1 mM) and 50 μ L of L-tyrosine (2 mM), dissolved in 20 mM phosphate buffer (pH 6.8). DMSO or test sample (50 μ L) was added into these mixtures in a 96-well microplate, followed by mixing with 50 μ L of mushroom tyrosinase solution (480 units/mL). After incubation at 25°C for 10 min, the amount of dopachrome in the reaction mixture was determined. The inhibitory activity of the sample was expressed as percentage to control based on the absorbance measured at 492 nm [6].

2.10. Determination of Free Radical Scavenging Activity. The effect of KA and KA esters on the free radical scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) activities was estimated on a 96-well plate [18]. Test compounds (100 μ L) dissolved in dimethyl sulfoxide solutions were added into 100 μ L of 0.2 mM DPPH in ethanol. 100 μ L of dimethyl sulfoxide solution alone was added into 100 μ L of 0.2 mM DPPH in ethanol as blank. The plates were incubated at 25°C for

30 min and the absorbance was read at 492 nm. The percentage of antioxidative activity was calculated according to (1),

Percentage of antioxidative activity

$$= \left[\frac{\text{absorbance of control} - \text{absorbance of test compound}}{\text{absorbance of control}} \right] \times 100. \quad (1)$$

DPPH is a stable free radical with violet colour. It turns to yellow in the presence of antioxidant and scavenging agents.

2.11. Statistical Analysis. Data were collected as mean \pm standard error (S.E.M) of at least three determinations. Statistical analysis was performed using Microsoft Excel 2007 (Microsoft, WA, USA). The evaluation of statistical significance was performed by Student *t*-test (two-tailed). $P < 0.05$ was considered statistically significant, $n \geq 3$, [2, 21, 24–28].

3. Results

3.1. Production of Kojic Acid Esters. The maximum yield of KA esters synthesized by enzymatic esterification using immobilized lipase is shown in Table 1. The structure of KAMO has been identified and characterized in our previous study [12, 17]. The yield for KAMO, KAML, and KAMP was 32.86%, 34.89%, and 29.30%, respectively. The time taken to reach a maximum KAMO, KAML, and KAMP concentration was 42 h, 15 h, and 12 h, respectively. Meanwhile, KAML and KAMP were identified using GC-MS where the purified samples were fragmented to simple ions. The MS data for the esterification of KA and palmitic acid showed that ions at m/z 141 and 365 arise from path a and b cleavage. The m/z 363 is the expected rearrangement ion resulting path c cleavage with loss of OH (m/z 480–17). The fragmentation at m/z 255 and 125 confirm path d and e cleavage. The product of the esterification, KAMP, is shown at m/z 380. Meanwhile, the MS data for the esterification of KA and lauric acid showed that ions at m/z 141 and 309 arise from path a and b cleavage. The m/z 307 is the expected rearrangement of ion resulting to path c cleavage with loss of OH (m/z 480–17). The fragmentation at m/z 199 and 125 confirm path d and e cleavage. The fragmentation at m/z 197 is the expected ion from path f cleavage. The data for esterification reaction of KA also showed that ions at m/z 142. The esterification product, KAML, is shown at m/z 324 (Figure 1).

The ¹H-NMR spectrum of the KAMP and KAML gave 3-hydrogen triplet at δ 0.88, indicating a terminal methyl group. Two hydrogen methylene signals are observed at H3'–H15' and H3'–H11' of KAMP and KAML, respectively. The downfield methylene signal at δ 2.40 was due to the present of CH₂ group, next to the ester linkage. The fatty acid chain was thus established to be present in the product. The KA portion of the molecule was confirmed by the presence of two singlet signals at δ 6.49 and δ 7.85 which were assigned to H-3 and H-6. H-7 gave a singlet signal at δ 4.93. The ¹³C-NMR spectrum gave a total carbon count for KAMP and KAML of 22 and 18, respectively. The C-1' (ester group) peak

TABLE 1: The yield of KA esters synthesized by enzymatic esterification using immobilized lipase from *Rhizomucor miehei* at optimal reaction condition.

Compound	*Time (h)	Temperature (°C)	Variables		Yield (%)
			Enzyme (g)	Substrate molar ratio (mmol)	
KAMO	42	52.5	0.33	0.5	32.86
KAML	15	50.0	0.20	5	34.89
KAMP	12	50.0	0.20	5	29.30

*A time of enzymatic esterification to achieve maximum concentration of KA esters.

Percentage yield was calculated using following equation:

$$\text{Yield (\%)} = (C_{\text{comp}}/\text{mole of KA}) \times \text{dilution factor} \times 100$$

$$C_{\text{comp}} = (A_{\text{comp}}/A_{\text{IS}}) \times (C_{\text{IS}}/D_{\text{RF}})$$

where, A_{comp} : area for each component; A_{IS} : area for internal standard; C_{IS} : concentration for internal standard; D_{RF} : D_{RF} standard/ D_{RF} internal standard; C_{comp} : concentration for each component.

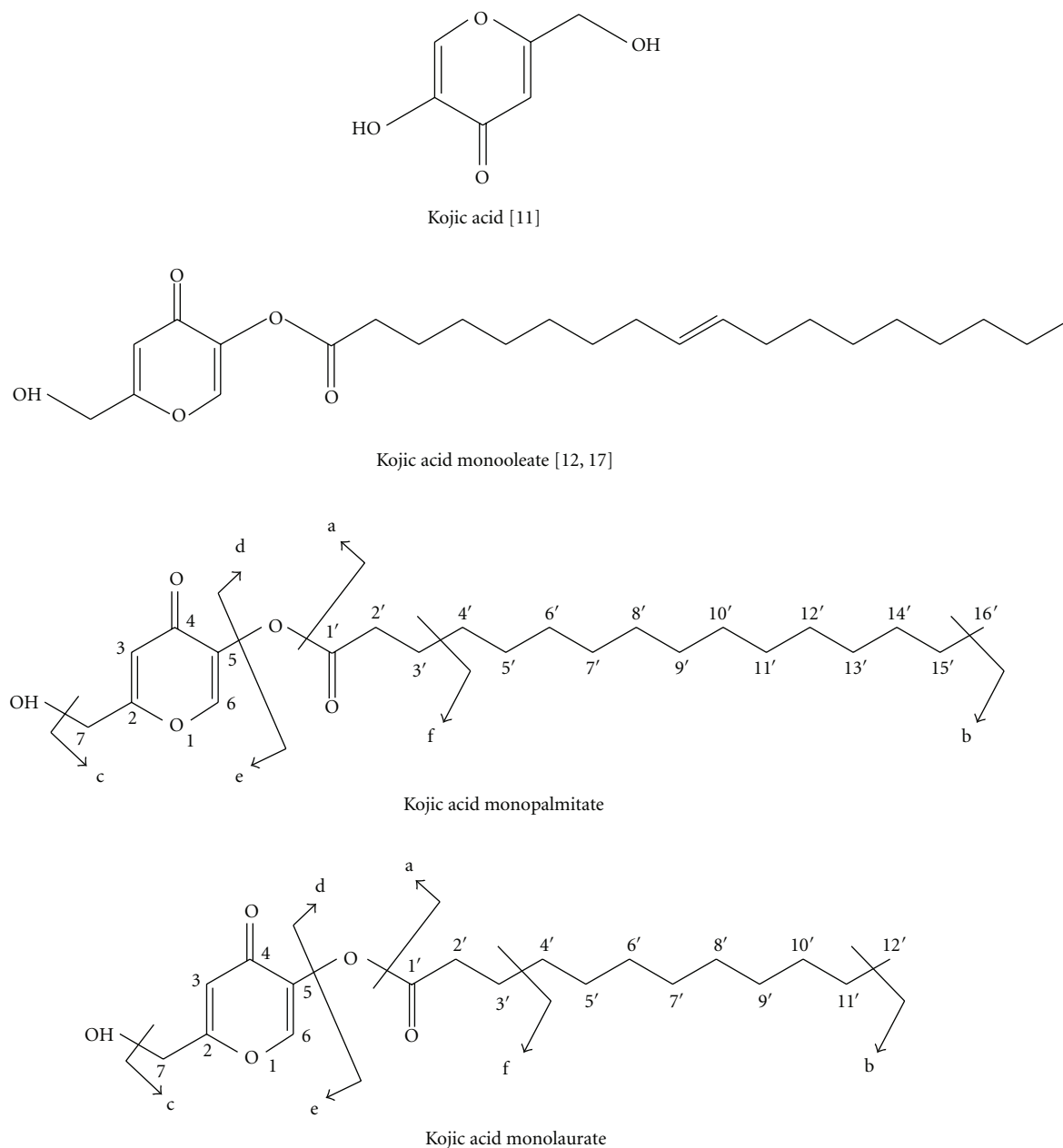


FIGURE 1: Chemical structure of kojic acid (KA), kojic acid monooleate (KAMO), kojic acid monopalmitate (KAMP) and kojic acid mono-laurate (KAML).

appeared at 163. Very low field signals were observed at δ 172 and δ 173 which were due to C-2 and C-4 of the pyrone ring. The other carbon assignments are also shown in Table 2.

The product formation and reactant disappearance were monitored by IR spectroscopy. The infrared spectrum of the KAMP showed the stretching of CH_2 and CH_3 which gave absorption peaks at 2848 cm^{-1} and 2914 cm^{-1} , respectively. The $\text{C}=\text{O}$ stretching for the expected ester carbonyl gave an absorption peak at 1695 cm^{-1} . The present of aromatic ring gave absorption at 1619 cm^{-1} . The CH_2 and CH_3 bends showed absorptions at 1458 cm^{-1} and 1290 cm^{-1} . Meanwhile, $\text{O}=\text{C}-\text{O}$ stretching absorbed at 1109 cm^{-1} . On the other hand, the infrared spectrum of the KAML showed the stretching of CH_2 and CH_3 which gave absorption peaks at 2849 cm^{-1} and 2915 cm^{-1} , respectively. The $\text{C}=\text{O}$ stretching for the expected ester carbonyl gave an absorption peak at 1693 cm^{-1} . The present of aromatic ring gave absorption at 1616 cm^{-1} . The CH_2 and CH_3 bends showed absorptions at 1427 cm^{-1} and 1292 cm^{-1} , respectively. Meanwhile, $\text{O}=\text{C}-\text{O}$ stretching absorbed at $1138-11073\text{ cm}^{-1}$.

3.2. Cytotoxicity Effect of KA and KA Esters. The results of cell viability assay using MTT in B16F1 melanoma cells are shown in Figure 2. There was no significant reduction of cell viability after incubation of pigmented B16F1 melanoma cells with KAMO, KAML, KAMP and KA at doses ranging from $7.81\text{ }\mu\text{g/mL}$ to $31.25\text{ }\mu\text{g/mL}$. However, the number of viable cells was significantly reduced to below 60% at KA concentration of $125\text{ }\mu\text{g/mL}$ and $500\text{ }\mu\text{g/mL}$. On the other hand, even at very high dosages of KAMO and KAMP ranging from 125 to $500\text{ }\mu\text{g/mL}$, more than 90% of B16F1 melanoma cells were still viable. Meanwhile, it was also noted that the number of viable cells was significantly reduced at 5% of DMSO which was used as a negative control.

3.3. Inhibitory Effect of KA and KA Esters on Melanin Content. The inhibitory effect of KA and KA esters on melanin formation in B16F1 melanoma cells treated with α -MSH is summarized in Figure 3. The inhibitory effect of KAMO, KAML, KAMP, and KA was evaluated at nontoxic doses ranging from 1.95 to $62.5\text{ }\mu\text{g/mL}$. The melanin content was significantly reduced at KA and KA esters concentration ranging from 31.3 to $62.5\text{ }\mu\text{g/mL}$. KA and KA esters showed similar melanin inhibitory effect at the lowest dose tested in this study ($1.95\text{ }\mu\text{g/mL}$). Even at the highest dose tested, KAML was found to have similar melanin inhibitory effect to KA. However, KAMP have slightly higher inhibitory effect than other compounds tested at doses ranging from $15.63\text{ }\mu\text{g/mL}$ to $62.5\text{ }\mu\text{g/mL}$.

3.4. Inhibitory Effect of KA and KA Esters on Cellular and Mushroom Tyrosinase Activity. The inhibitory effect of KA and KA esters in B16F1 melanoma cells is summarized in Figure 4. The inhibitory effect of KA and KA esters was evaluated at nontoxic doses, ranging from 1.95 to $62.5\text{ }\mu\text{g/mL}$. Incubation of pigmented melanoma B16F1 melanoma cells with KA and KA esters at doses ranging from $31.25\text{ }\mu\text{g/mL}$ to

TABLE 2: $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ data for KAMP and KAML.

Carbon no.	KAMP		KAML	
	δ ^1H	δ ^{13}C	δ ^1H	δ ^{13}C
C-2	—	172.69	—	172.69
C-3	6.49 (s)	110.99	6.49 (s)	110.94
C-4	—	173.85	—	173.82
C-5	—	137.75	—	137.67
C-6	7.85 (s)	145.80	7.85 (s)	145.77
C-7	4.93 (s)	61.13	4.93 (s)	61.13
C-1' ester group	—	163.11	—	163.13
C-2'	2.40 (t)	33.88	2.40 (t)	33.88
C-3'	1.66 (m)	24.77	1.64 (m)	24.77
C-4'	1.29	29.18	1.28	29.18
C-5'	1.29	29.34	1.28	29.23
C-6'	1.29	29.41	1.28	29.30
C-7'	1.29	29.56	1.28	29.41
C-8'	1.29	29.64	1.28	29.56
C-9'	1.29	29.65	1.28	29.06
C-10'	1.29	29.67	1.28	31.89
C-11'	1.29	29.67	1.30	22.66
C-12'	1.29	29.64	0.88 (t)	14.09
C-13'	1.29	29.06		
C-14'	1.29	31.91		
C-15'	1.30	22.67		
C-16'	0.88 (t)	14.09		

$62.5\text{ }\mu\text{g/mL}$ showed significant reduction in cellular tyrosinase activity. At very low doses of KA and KA esters, ranging from 1.95 to $15.25\text{ }\mu\text{g/mL}$, only a slight reduction in cellular tyrosinase activity was observed. The inhibitory effect of KA and KA esters on cellular tyrosinase activity at doses ranging from 31.25 to $62.5\text{ }\mu\text{g/mL}$ was not significantly different. At the same dose ($15.63\text{ }\mu\text{g/mL}$), KAML and KAMP reduced cellular tyrosinase activity at a greater extent than KAMO.

Inhibitory effect of KA and KA esters on mushroom tyrosinase activity is illustrated in Figure 5. The inhibitory effect of KA and KA esters was evaluated at doses ranging from 3.91 to $250\text{ }\mu\text{g/mL}$. In this study, mushroom tyrosinase inhibitory was found to be in dose-dependent manner. Among KA esters, KAMO significantly inhibited mushroom tyrosinase superior than KAML and KAMP. The inhibitory effect of mushroom tyrosinase activity of KAMO was not significantly different to KA at doses ranging from 62.5 to $250\text{ }\mu\text{g/mL}$.

3.5. The Antioxidant Activity of KA and KA Esters. The correlation between antimelanogenic activity with oxidative properties of KA and KA esters was also investigated. KA and KA esters showed mild free radical scavenging activity at concentrations ranging from 1.95 to $1000\text{ }\mu\text{g/mL}$ (Table 3). 2,2-diphenyl-1-picrylhydrazyl (DPPH) generation was inhibited by KA and KA esters in dose dependent manner. Among them, KA and KAMO showed slightly better antioxidant activity at higher doses ($1000\text{ }\mu\text{g/mL}$) as compared to KAML and KAMP. L-ascorbic acid, a well-known antimelanogenic

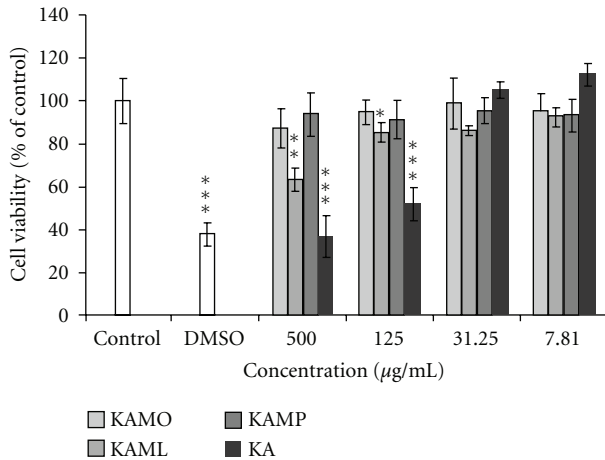


FIGURE 2: The effects of KAMO, KAML, KAMP, and KA on the viability of B16F1 melanoma cells. DMSO (5%) was used as a negative control. Cells were treated with various doses of KAMO, KAML, KAMP, and KA (7.81–500 $\mu\text{g}/\text{mL}$) for a total of 72 h incubation and were examined by MTT assay. Denotes $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to untreated control. Data are presented as means \pm S.E.M and expressed as % of control, $n \geq 3$.

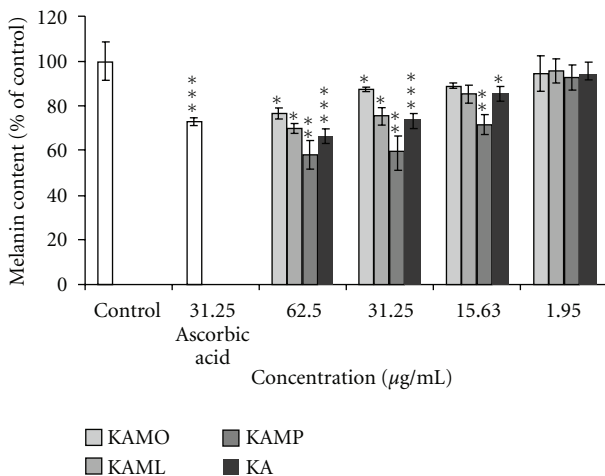


FIGURE 3: The effects of KAMO, KAML, KAMP, and KA on melanin content in B16F1 melanoma cells. Denotes $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to α -MSH treated control. Data are presented as means \pm S.E.M, and expressed as % of control. 31.25 $\mu\text{g}/\text{mL}$ ascorbic acid was used as reference. $n \geq 3$.

vitamin and antioxidant, exerted more scavenging activity than KA and KA esters at 62.5 $\mu\text{g}/\text{mL}$.

4. Discussion

B16F1 melanoma cells is a widely used model to evaluate depigmentation activity with high level of tyrosinase and melanin content as compared to B16F10, due to high level of *msg1* (melanocyte-specific gene) [22, 24, 28–33]. In melanogenesis, tyrosinase-related protein-2 and tyrosinase-related protein-1 catalyzes conversion of DOPachrome to DHICA

TABLE 3: Antioxidant effects of KA, KAMP, KAML and KAMO using DPPH. Denotes $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to untreated control. Data are presented as means \pm S.E.M, and expressed as % of control, $n \geq 3$.

Sample	Concentration ($\mu\text{g}/\text{mL}$)	Scavenging activity (% of control) S.E.M
Control	0	0.06 \pm 0.06
	1.95	5.86 \pm 0.28***
	62.5	29.24 \pm 4.18*
	250	46.73 \pm 5.21*
	1000	57.07 \pm 1.64***
KA	1.95	3.00 \pm 0.40*
	62.5	4.69 \pm 0.34**
	250	9.41 \pm 3.43
	1000	53.05 \pm 13.47*
	KAMP	1.95
62.5		11.96 \pm 9.61
250		16.91 \pm 5.06
1000		44.70 \pm 6.66*
KAML		1.95
	62.5	24.54 \pm 2.89**
	250	35.20 \pm 1.41**
	1000	59.01 \pm 2.89**
	KAMO	62.5
250		35.20 \pm 1.41**
L-ascorbic	62.5	48.73 \pm 3.84***

and oxidation of DHICA, respectively, to form melanin [34]. In the presence of alpha-melanocyte stimulating hormone (α -MSH), and isobutylmethylxanthine (IBMX), B16 melanoma cells expressed great amount of tyrosinase and melanin synthesis [10, 31]. α -MSH binds to melanocortin receptor (MC1R), resulting in the activation of stimulatory GTP-binding protein (Gs), which in turn, stimulates adenylate cyclase to generate cAMP. cAMP increases melanin synthesis via activation of cAMP-dependent protein kinase (PKA) and microphthalmia-associated transcription factor (MITF), a melanocyte-specific transcription factor, leading to induction of tyrosinase expression [35–38]. Hyperpigmentation and melasma are the result from the accumulation of tyrosinase and melanin in cells. Therefore, the ability of KAMO, KAML, and KAMP to inhibit tyrosinase activity and melanin content in alpha-MSH induced B16F1 melanoma cells showed their potential as depigmenting agent and to treat hyperpigmentation in vitro.

Melanin synthesis can also be induced by the presence of free radicals and reactive species. Excessive exposure of ultraviolet radiation, metal ions, free radicals, and reactive species have significantly stimulate transcription of tyrosinase gene and contribute to hyperpigmentation [4, 39]. The presence of metal ions, free radical, and reactive species caused oxidation in melanogenesis pathway that will result in high melanin synthesis [8]. Antioxidant such as vitamin C and multivitamin were known to scavenge free radicals and inhibit tyrosinase activity [40]. The stable DPPH free radicals are a commonly use model and technique to evaluate antioxidant activities. The effect of antioxidants on DPPH free radicals was due to their hydrogen donating ability. DPPH free radicals accept electron or hydrogen radicals to become

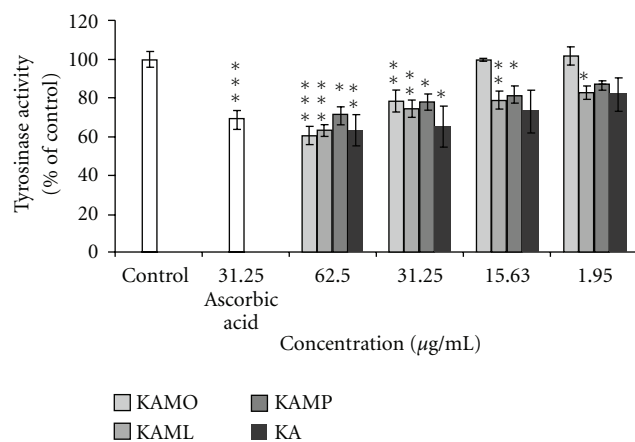


FIGURE 4: The results of the inhibition of tyrosinase activity by KAMO, KAML, KAMP, and KA in B16F1 melanoma cells. Denotes * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to α -MSH treated control. Data are presented as means \pm S.E.M, and expressed as % of control. 31.25 $\mu\text{g/mL}$ ascorbic acid was used as reference. $n \geq 3$.

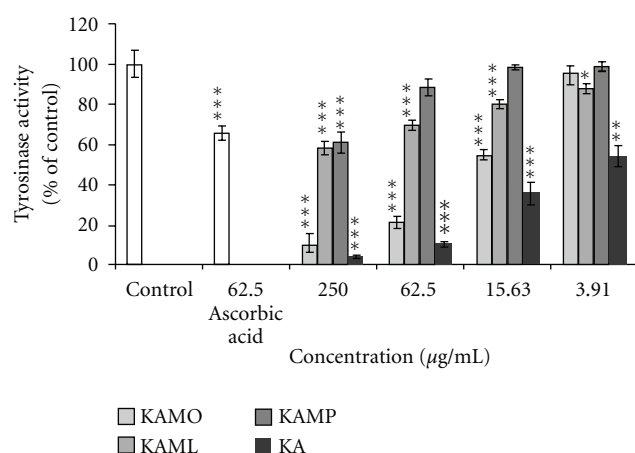


FIGURE 5: The results of the inhibition of mushroom tyrosinase activity by KAMO, KAML, KAMP, and KA. Denotes * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to α -MSH treated control. Data are presented as means \pm S.E.M, and expressed as % of control. 62.5 $\mu\text{g/mL}$ ascorbic acid was used as reference. $n \geq 3$.

stable diamagnetic molecules. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation [41]. Therefore, the potency of hydroxyl group (OH) at C-7 of KA esters to stabilize free radicals and chelate metal ions [9] may help to reduce melanogenesis process and down-regulate hyperpigmentation. In this study, KA esters synthesized using RMIM have slightly greater scavenging activity than other KA esters that were previously reported in the literature [14]. Tyrosinase is known as copper-containing enzyme, thus the capability of KA and KA esters to chelate metal ions may chelate copper in tyrosinase, changing its three-dimensional conformation to inhibit its enzymatic activity [11].

The cytotoxicity of KA and KA esters was investigated in this study. It was previously suggested that inhibition of upregulated tyrosinase enzyme in melanoma cells might inhibit cell proliferation of melanoma cells [42, 43]. This is due to the correlation of microphthalmia-associated transcription factor (MITF) and extracellular signal-regulated kinase (ERK) in the pigmentation, proliferation, and survival of melanocytes and melanoma [23, 26, 35]. Due to this reason, it was expected that the inhibition of MITF expression may also inhibit melanoma cells proliferation. However, KA and KA esters were only known to inhibit melanogenesis by direct inhibition to tyrosinase and do not inhibit the expression of the transcription factor [5]. Therefore, the melanoma cells can still proliferate but the tyrosinase produced are not functional due to inhibition of KA and KA esters. In another study, α -melanocyte-stimulating hormone (MSH) decreased a critical mediator in the tumorigenesis (syndecan-2 expression), melanoma cell migration, and invasion in a melanin synthesis-independent manner [44]. Other depigmenting compound like hydroquinone, is a strong tyrosinase inhibitor with bleaching effect and exerted very high cytotoxicity at high concentrations [45]. Besides that, vitamin C and multivitamin showed satisfactory inhibitory effect in melanin content and tyrosinase activity at low concentrations, though it could be toxic at high concentrations [40]. KA was claimed to be nontoxic at doses below 100 $\mu\text{g/mL}$ [18, 27, 46]. KA esters derived in this study have very low cytotoxicity, even at very high doses (up to 500 $\mu\text{g/L}$). In summary, results from this study indicated that KA and KA esters are potential depigmenting agents with low cytotoxicity for application in cosmetic and skin-care products.

5. Conclusion

KA esters derived from esterification of KA and palm oil based fatty acid have been demonstrated as a safe and nontoxic depigmenting agents with a satisfactory inhibitory effect on melanin formation and tyrosinase activity as determined on α -MSH induced B16F1 melanoma cells. Thus, it can be suggested that these depigmenting compounds have potential to be used in cosmetic formulations and to treat hyperpigmentation.

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