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ORIGINAL PAPER

Ficus deltoidea (Mas cotek) extract exerted anti-melanogenic activity by preventing tyrosinase activity in vitro and by suppressing tyrosinase gene expression in B16F1 melanoma cells

Myoung-Jin Oh · Mariani Abdul Hamid · Sulaiman Ngadiran · Young-Kwon Seo · Mohamad Roji Sarmidi · Chang Seo Park

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Abstract *Ficus deltoidea* (Mas cotek) water extract has been widely used for woman health in Malaysia. Our investigation focused to identify anti-melanogenic efficacy of *F. deltoidea* since it has been known to have strong anti-oxidant activities. Anti-melanogenic effect of *F. deltoidea* extract was analyzed using cultured B16F1 melanoma cells. Cytotoxicity of the extract was measured using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and determined the highest concentration of the extract that did not affect cell viability as 0.1% (w/v). α-MSH-induced melanin synthesis was significantly inhibited with dose-dependent manner by treatment of *F. deltoidea* leave extract, which was comparable to that of kojic acid. The extract directly inhibited mushroom tyrosinase activity and intracellular tyrosinase activity of B16F1 as well. The inhibition of intracellular tyrosinase activity was found to be exerted at the protein expression level when analyzed by immunoblot and tyrosinase zymography. The expression of microphthalmia-associated transcription factor (MITF) was also reduced by the *F. deltoidea* extract. In conclusion, *F. deltoidea* extract has strong anti-melanogenic activity that is exerted by direct inhibition of tyrosinase enzyme activity and by down-regulation of the expression of genes involved in the melanogenesis pathways. Collectively, data shown in this study strongly suggest that *F. deltoidea* extract has potential to be used as a novel depigmenting agent for cosmetics.

Keywords *Ficus deltoidea* · Melanogenesis · Melanin · Tyrosinase · MITF

Abbreviations

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide
α-MSH	α-Melanocyte-stimulating hormone
MITF	Microphthalmia-associated transcription factor
POMC	Proopiomelanocortin
MC1R	Melanocortin-1 receptor
PKC	Protein kinase C
ACTH	Adrenocorticotropic hormone
ATP	Adenosine triphosphate
cAMP	Cyclic Adenosine mono-phosphate
CREB	cAMP response element binding protein
CRE	cAMP response element
DCT	Dopachrome tautomerase
L-dopa	L-3,4-Dihydroxyphenylalanine
b-HLH	Basic-helix-loop-helix
TRP	Tyrosinase-related protein
DPPH	2,2-Diphenyl-1-Picrylhydrazyl

Introduction

Melanogenesis is a biosynthetic pathway for melanin production from melanocytes located in the lowest layer of

M.-J. Oh · M. Abdul Hamid · C. S. Park (✉)
Department of Chemical and Biochemical Engineering, Dongguk University, 3-26, Pil-dong, Chung-gu, Seoul 100-715, Korea
e-mail: dgucsp@dongguk.edu

M. Abdul Hamid · S. Ngadiran · M. R. Sarmidi
Chemical Engineering Pilot Plant, Universiti Teknologi Malaysia, 81310 Skudai, Johor Bahru, Johor, Malaysia

Y.-K. Seo
Dongguk University Research Institute of Biotechnology, Dongguk University, 3-26, Pil-dong, Chung-gu, Seoul 100-715, Korea

epidermis in human skin [39]. UV irradiation, a representative melanogenesis triggering factor, causes secretion of several melanogenic signaling delivery substances such as proopiomelanocortin (POMC), a precursor of α-melanocyte-stimulating hormone (α-MSH), a receptor of α-MSH, melanocortin-1 receptor (MC1R), melanogenic enzymes, tyrosinase, tyrosinase-related protein 1 (TRP1) and protein kinase C (PKC) from melanocytes [35]. In human epidermis, once α-MSH and adrenocorticotropic hormone (ACTH) are produced and released by keratinocytes after UV irradiation [31], they combine with their specific receptor MC1R and activate adenyl cyclase through G-protein which converts adenosine triphosphate (ATP) to cyclic adenosine mono-phosphate (cAMP) leading to increase of intracellular cAMP level [2, 7]. cAMP generated from ATP, plays a role as a second messenger in intracellular signaling pathway [25]. When increased cAMP has effect on PKA, cAMP response element binding protein (CREB) and cAMP response element (CRE) located in M promoter of microphthalmia-associated transcription factor (MITF) gene bond together [34]. Increased expression of MITF leads to enhancement of the expression of genes for melanogenic enzyme family, tyrosinase, tyrosinase-related protein 1, dopachrome tautomerase (DCT), tyrosinase-related protein 2, which eventually stimulates melanin synthesis [3, 5, 8]. cAMP-related biological effects depend on protein kinase A (PKA) and once PKA phosphorylates CREB, it interacts with CRE and increases the expression of MITF gene-containing CRE responsible DNA sequence in its promoter region [25]. Therefore, phytochemicals and other bio-materials that can modulate intracellular cAMP level might also be able to regulate melanogenesis in human and mouse melanocytes [32].

The skin pigmentation processes involve de novo synthesis of melanin in melanocytes and transfer of the synthesised melanin packed in melanosome to neighboring keratinocytes, which eventually turns the skin color into dark [12, 29]. Melanosome contains three types of enzymes, tyrosinase, TRP1, DCT (TRP2) [18]. Tyrosinase is a rate-limiting enzyme involved in melanin synthesis that hydroxylates tyrosine, a kind of phenylalanine, to L-3, 4-dihydroxyphenylalanine (L-DOPA) and oxidizes L-DOPA to DOPA quinone [17]. Excessive accumulation of DOPA quinone generated from hydroxylation and oxidation of tyrosine forms DOPA chrome, which conditionally exhausted cysteine, resulted in accumulation of black and brownish pigment eumelanin. Another type of melanin, pheomelanin is produced through formation of 3 or 5 cysteinyl DOPA on condition of being existence of cysteine [17, 22, 38]. These three enzymes determine types of melanin to eumelanin or pheomelanin. Accordingly, our skin color can be determined by a ratio between the two types of melanin, the amount of each type of melanin and the extent of transferring melanosomes to keratinocytes [8]. MITF which has basic-helix–loop–helix (b-HLH) and b-HLH-leucine zipper motif, is a key transcription factor critical for melanogenic gene expression and has an essential role for regulation at the transcription level in melanogenesis of tyrosinase, TRP1 and TRP2 [23, 36]. MITF can bind to the M-box only as the phosphorylated form and stimulate expression of the target genes. Therefore, dephosphorylation of MITF leads to degradation of the protein that eventually prevents melanogenesis [13, 24]. Recently, it have been reported that MITF is also involved in formation and transfer of melanosomes [26, 37].

Extensive efforts have been made to search for novel depigmenting agents with little success. In industrial point of view, cosmetics are able to help depigment skin color which is one of the important parts among several functional cosmetic fields. Especially, development of novel depigmenting phytochemicals from natural sources has become new popular trends recently. These depigmenting materials could be applied to melanogenic disorders, melasma along with whitening cosmetic products [33, 35]. Malaysia's tropical rain forest is unique with large biodiversity of valuable natural resources. *F. deltoidea* (Mas cotek) and *Labisia pumila* are two of the best-known local traditional medicinal herbs and have been used for a long time by women who is preparing or after giving birth. More specifically, these plants were used as a postpartum treatment to help in contracting the muscles of the uterus and in the healing of the uterus and vaginal canal. Recently, they began to slowly receive international recognition for their medicinal values and health benefits. *F. deltoidea* is a popular medicinal herb in Malaysia [30, 32]. *F. deltoidea* has been scientifically investigated by two local Malaysia institutions, the University Malaya and the Malaysian Planting Research Institute (MARDI). Research result showed that *F. deltoidea* possesses five active components which are required by the human body namely flavanoid, tannins, triterpenoids, proanthocyanins and phenols. These active components are medically shown to assist in human memory and were used by doctors to treat patients with disabilities in concentration. In addition, *F. deltoidea* helps to assist the effectiveness of vitamin C in controlling nitric oxide and blood circulation. *F. deltoidea* protects the heart in three ways, prevent blood clot, control LDL (bad cholesterol) oxygenation, and reduce blood pressure. The leaves of male and female plants are mixed in specific proportions to be taken as an aphrodisiac. Among the traditional practices, *F. deltoidea* has been used for regulating blood pressure, increasing and recovering sexual desire, womb contraction after delivery, reducing cholesterol, reducing blood sugar level, treatment of migraines, toxin

removal, delay menopause, nausea, joints pains, piles pain, and improving blood circulation. On the other hand, skin physiological efficacies of *Ficus deltoidea* have not been reported. Several researchers have also reported that *F. deltoidea* has strong anti-oxidant activity [14]. These findings suggest that *F. deltoidea* might have anti-melanogenic activity by prohibiting the process of tyrosinase oxidation [11]. Our investigation was made as the first attempt to find out novel whitening material from undeveloped resource which was motivated by strong anti-oxidant effect of *F. deltoidea*.

Materials and methods

Materials

3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), kojic acid, arbutin, α-MSH, dimethyl sulfoxide (DMSO), mushroom tyrosinase were purchased from Sigma–Aldrich (St.Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin were obtained from Welgene (Daegu, Korea). Antibodies against tyrosinase, MITF, and β-actin were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Fresh leaves of *F. deltoidea* at the mature stage were purchased from Toko Tenaga Keluarga Sdn Bhd, Johor, Malaysia. The leaves selected for the study is from the division of Magnoliophyta, class of Magnoliopsida, family of Moracea, genus of *Ficus* and species of *F. deltoidea*. The selected diameter of the leaves is 6 ± 2 cm from all 100 trees. The entire leaves were carefully washed in portable water and immediately dried at a temperature of 45–50°C in a vacuum dryer for 8 h and stored at a room temperature for future use. The dry leaves were further grind using a Stainless Steel Powder Grinder PG-10 (SUS), driving motor 10 HP with fineness 100 meshes.

Extraction of *Ficus deltoidea* leaves

Extraction of *F. deltoidea* leaves was performed as follows: 5 kg of dried *F. deltoidea* leaves was extracted in 80 l of boiling water for 2 h. After removal of the solid parts by filtration, the extracted solution was spray-dried using pilot spray dryer (Niro A/S, GEA Group, Soeborg Denmark). The inlet and outlet temperature were 200°C and 110°C, respectively, with feed capacity of 7 l/hr. The yield of extract was obtained at 8% of total raw material. The resultant powder was used for determination of anti-melanogenesis effect. Working solutions of the sample was prepared as follows: dried powder was dissolved in water to a final concentration of 1% (w/v) and sterilized through filtration. The resulting filtrate was stored at −20°C.

Cell culture

B16F1 melanoma was obtained from Korea Cell Line Bank (KCLB) and sustained in 10% FBS and 1% penicillin–streptomycin supplemented DMEM. Incubation was carried out in 5% CO_2 incubator at 37°C.

α-MSH and *Ficus deltoidea* treatment

B16F1 melanoma cells were seeded at a density of 1.5×10^5 cells/well in six well plates containing DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. After 24 h, the medium was substituted by fresh one supplemented with 5 nM of α-MSH and different concentrations of *F. deltoidea* extract with the minimum concentration 0.0125% (equals to 0.125 mg/ml) and incubated for 48 h. α-MSH untreated cells were used as a negative control and 800 μM of kojic acid (equals to 0.114 mg/ml) and 2 mM of arbutin (equals to 0.136 mg/ml) were used to compare with the results of *F. deltoidea*.

MTT assay

The MTT assay is colorimetric assays for measuring the mitochondrial activity of enzymes that reduce MTT to formazan dyes, giving a purple color. A main application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth. After 48 h, the culture medium was removed and incubated with MTT solution at 37°C for 90 min. The solution was replaced with 0.04 N HCl–isopropyl alcohol solution, and further incubation was added at room temperature for 30 min. Harvested solution was centrifuged at 13,000 rpm for 5 min. Absorbance of supernatant was measured at 570 nm using microplate reader (Perkin Elmer, USA).

DPPH assay

Scavenging of DPPH free radical is the basis of a common antioxidant assay. A number of protocols have been followed for this assay resulting in variation in the results of different laboratories. DPPH is a common abbreviation for an organic chemical compound 2,2-diphenyl-1-picrylhydrazyl (DPPH). It is a dark-colored crystalline powder composed of stable free-radical molecules. DPPH has two major applications, both in laboratory research: one is a monitor of chemical reactions involving radicals and another is a standard of the position and intensity of electron paramagnetic resonance signals. The method with DPPH as a stable free radical to measure radical-scavenging activity

has been widely used. Antioxidants react with DPPH, which is a stable free radical, and convert it to 1,1-diphenyl-2-(2,4,6- trinitrophenyl) hydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant compounds. DPPH was used to measure radical erasure capacity (anti-oxidative property) of various agricultural products. In this assay, DPPH (Sigma, USA) was dissolved in ethanol to a volume of 250 μM followed by sonication for 5 min to obtain the stable free radical DPPH (4). The test compound was diluted in DPPH solution in a ratio of 1:1. Appropriate controls were run in each series and fresh DPPH solution was prepared daily. *F. deltoidea* extract was tested in triplicate at five different concentrations such that a 50% decrease in DPPH absorbance can be calculated. Absorbance of the reaction mixture was measured after 20 min using a UV/Vis spectrometer (DU730, Beckman Coulter, USA). The FSC_{50} (concentration causing 50% radical-scavenging activity) value of *F. deltoidea* extract was determined and compared with L-ascorbic acid (Sigma, USA) and Trolox (Sigma, USA).

Melanin assay

Secreted melanin assay was performed as described previously with slight modifications [21]. After 48 h, the culture medium was harvested and centrifuged at 10,000 rpm for 10 min. Absorbance was measured at 405 nm using microplate reader (Perkin Elmer, USA). The secreted melanin was expressed in a ratio calculated as follows:

$$[\text{Secreted melanin } (\%) = (C - A)/B \times 100].$$

In the calculation, A represents absorbance value of medium supplemented with each samples (before incubation), B represents absorbance value of the α-MSH untreated medium (harvested after 48 h incubation) and C represents absorbance value of medium supplemented with each samples (harvested after 48 h incubation). All values were obtained from measurement at 405 nm.

Mushroom tyrosinase inhibition assay

In vitro mushroom tyrosinase inhibition assay was performed as described previously with slight modifications [16]. 140 μl of *F. deltoidea* extract was prepared at appropriate concentrations using 10 mM sodium phosphate buffer (pH 6.8) in a 96 well plate and 40 μl of 10 μg/ml mushroom tyrosinase in 10 mM sodium phosphate buffer was added to each well and incubated at room temperature for 10 min. Mixed with 20 μl of 10 mM L-DOPA solution in 10 mM Sodium phosphate buffer and incubated for 30 min. Absorbance was measured at 405 nm using microplate reader (Perkin Elmer, USA). The ratio of tyrosinase activity was calculated as follows [21].

$$[\text{Mushroom tyrosinase activity } (\%) = (C - A)/B \times 100]$$

In the calculation, A represents absorbance value of sodium phosphate buffer supplemented with each concentrations of *F. deltoidea* without mushroom tyrosinase and B represents absorbance value of the mushroom tyrosinase only treated sodium phosphate buffer and C represents absorbance value of sodium phosphate buffer supplemented with each concentrations of *F. deltoidea* with mushroom tyrosinase. All values were obtained from measurement at 405 nm.

Intracellular tyrosinase activity assay

Intracellular tyrosinase activity assay was performed as described previously with slight modifications [17, 28]. After 48 h, the culture cells were washed with PBS and harvested in RIPA cell lysis buffer supplemented with protease inhibitor. Cell lysate was obtained after centrifugation at 13,000 rpm for 15 min. The amount of protein was calculated by Bradford assay (Biorad, CA, USA) using BSA as a standard (bovine serum albumin, Biorad, CA, USA). The amount of each cell lysate was adjusted to give the same protein concentration with the lysis buffer. 10 μl of 10 mM of L-DOPA in sodium phosphate buffer was added and incubated for 1 h at room temperature. Absorbance was measured at 405 nm using microplate reader (Perkin Elmer, USA).

Tyrosinase zymography (L-DOPA staining)

Tyrosinase zymography was performed as described previously [9]. After 48 h, the culture cells were washed with PBS and harvested with RIPA cell lysis buffer supplemented protease inhibitor. The amount of protein amount was also equilibrated as in the intracellular tyrosinase assay. Each sample mixed with zymogram sample buffer was loaded on 8% gel SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). After running, the gel was soaked in 0.1 M sodium phosphate buffer for 30 min twice. Staining was carried out with 10 mM L-dopa in 0.1 M sodium phosphate buffer for 1 h at 37°C.

Immunoblot assays

Once the cell lysates were prepared using a standard protocol, each sample was mixed with laemmli sample buffer and denaturated at 85°C for 5 min. Thereafter, samples were loaded on 8% gel SDS-PAGE (sodium dodecyl sulfate poly acrylamide gel electrophoresis). After running, the gel was transferred to PVDF membrane and blocked with 5% skimmed milk. Anti-tyrosinase (Santa cruz, USA), anti-MITF (Santa cruz, USA) were used as primary antibodies

and anti-goat IgG-HRP, anti-mouse IgG-HRP (Santa cruz, USA) were used as secondary antibodies. The antigen–antibody reaction was detected using ECL solution system (Perkin Elmer, MA, USA).

Statistical analysis

Differences between results of the melanin assay, intracellular tyrosinase activity assay, mushroom tyrosinase assay were assessed for statistical significance using the Student's *t* test.

Results

Ficus extract has strong radical-scavenging activity

To evaluate anti-oxidant activity of *F. deltoidea* extract, DPPH assay was performed. The absorbance of the free-radical DPPH decreased when measured at 517 nm for five different concentrations and produced a color change in colorization from violet to yellow. The FSC_{50} value determined for *F. deltoidea* extract was 35.19 μg/ml (Table 1). The FSC_{50} value of vitamin C and Trolox were 27.62 and 29.57 μg/ml, respectively. The data indicates that *F. deltoidea* extract has strong anti-oxidant activity comparable to that of vitamin C.

Effect of *Ficus deltoidea* on cell mitochondrial activity using cultured B16F1 melanoma

Cellular mitochondrial activity of cultured B16F1 melanoma cell was measured by MTT assay method. As shown in Fig. 1, *F. deltoidea* had no detectable adverse effect on cellular mitochondrial activity at concentrations between 0.0125 and 0.05% (w/v). Cellular mitochondrial activity was slightly decreased when the concentration of *F. deltoidea* was 0.1%. Based on this observation, rest of the experiments was performed within these concentrations range.

Table 1 50% Free radical-scavenging activity mass content of antioxidants

Antioxidant	Scavenging activity (FSC^{c}_{50}) Concentration (μg/ml)[a]
L-Ascrobic acid	27.62 ± 1.05
Trolox	29.57 ± 1.71
Ficus (DMSO)[b]	35.19 ± 1.46

[a] Each value is the mean ± SD of three replicate assays

[b] Each () is the mean solvent of sample extract

[c] FSC_{50} is the mean 50% Free-radical scavenging activity

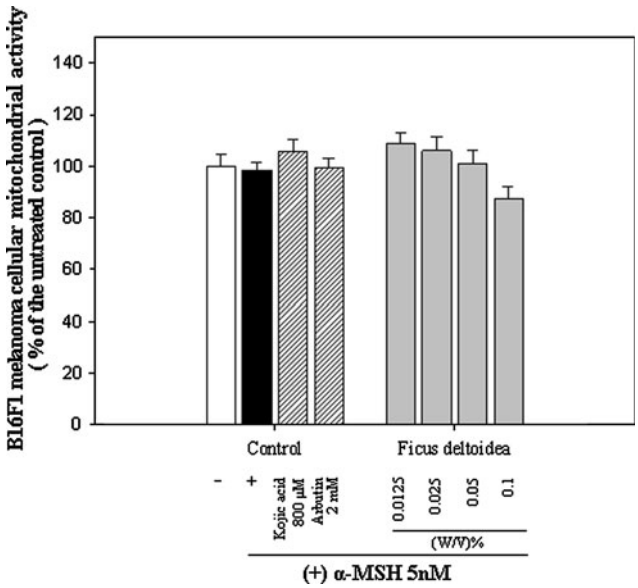

Fig. 1 *Ficus deltoidea* has no detectable influence on B16F1 melanoma cellular mitochondrial activity. B16F1 melanoma cellular mitochondrial activity was validated through MTT assay method. As shown in Fig. 1, B16F1 melanoma cellular mitochondrial activity was increased by *F. deltoidea* treatment ranges between 0.0125 and 0.05% (w/v). At the concentration of 0.1%, cellular mitochondrial activity was shown to be 90% and decreasing rate of cellular mitochondrial activity was grown according to the high concentration (data not shown). Each determination was made in triplicate and the data shown represent the mean ± SD

The inhibitory effect of *Ficus deltoidea* extract on α-MSH induced melanin synthesis

The effect of *F. deltoidea* extract on the melanin synthesis in B16F1 melanoma cells was investigated. The melanin contents of B16F1 cells treated with different concentrations of *F. deltoidea* extract were measured. Kojic acid and arbutin have been proved to be effective anti-melanogenic agents which were used as positive controls. Cells were treated with the *F. deltoidea* extract (0.0125, 0.025, 0.05, and 0.1%) or kojic acid (800 μM equals to 0.114 mg/ml) and arbutin (2 mM equals to 0.136 mg/ml) for 2 days and then the amount of melanin secreted into the culture medium and presented inside the cells were analyzed, respectively.

As shown in Fig. 2, the secreted melanin content increased more than fourfolds upon treated with α-MSH. Kojic acid clearly demonstrated its powerful anti-melanogenic activity by reducing the secreted melanin to the level of untreated control and arbutin also showed significant reduction of the melanin secretion. *F. deltoidea* extract showed even stronger inhibitory effect on the secretion of melanin than kojic acid in this experimental condition. Dose-dependent reduction of the secreted melanin content was observed when treated with increasing amount of

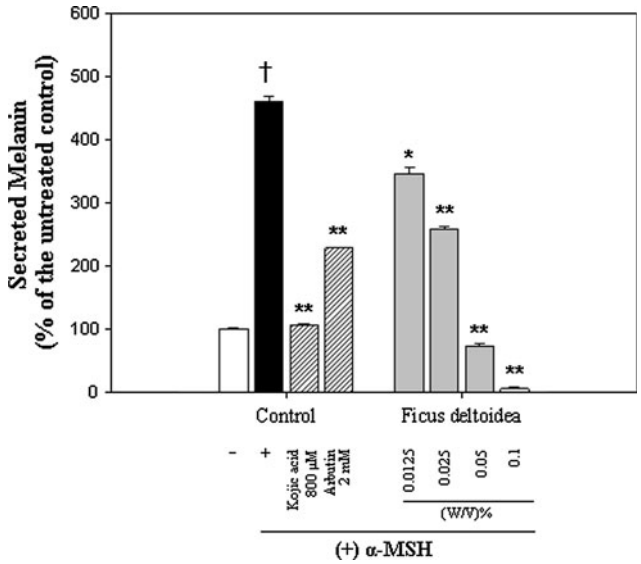

Fig. 2 α-MSH induced melanin synthesis was completely inhibited by *F. deltoidea* treatment. As indicated in Fig. 2, secreted melanin assay result presented that *F. deltoidea* strongly reduced α-MSH derived melanin secretion. Inhibited action of *F. deltoidea* at the concentration of 0.05% was fourfold decreased than α-MSH treated control and the result of 0.1% was more efficient than α-MSH non-treated control. This reduction efficacy was apparently effective than 800 μM of kojic acid or 2 mM of arbutin. Reduction of synthesised melanin was showed in a dose dependant manner. Results are the average of three independent experiments ± SD. $*P < 0.05$, $**P < 0.01$ compared to (+) α-MSH control

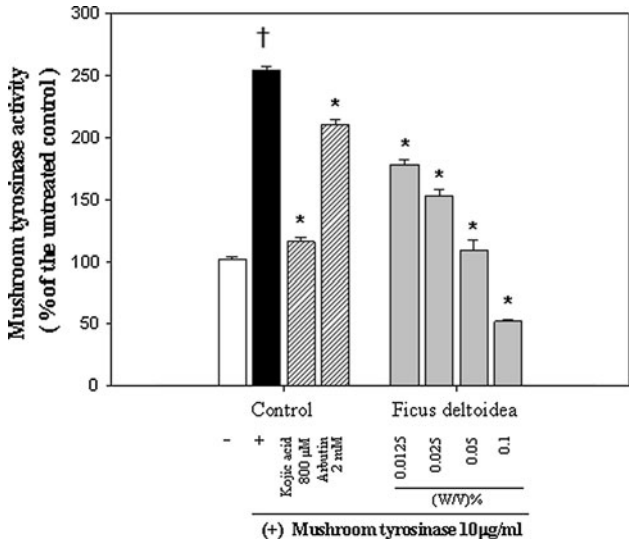

Fig. 3 *Ficus deltoidea* inhibited tyrosianse oxidation using Mushroom tyrosinase activity inhibition assay method. The process of mushroom tyrosinase oxidation using L-DOPA was prohibited by *F. deltoidea*. The treatment of *F. deltoidea* at 0the concentration of 0.1% presented more effective reducing effect than kojic acid or arbutin. Results are the average of three independent experiments ± SD. $*P < 0.01$ compared to (+) α-MSH control

F. deltoidea extract. The inhibitory effect of 0.05% of *F. deltoidea* extract on the secretion of melanin was similar to that of kojic acid. The melanin secretion almost abolished when the B16F1 melanoma cells was treated with 0.1% of the extract indicating that *F. deltoidea* extract might have suppressed the melanin synthesis in the cells.

Mushroom tyrosinase activity directly inhibited by *F. deltoidea* extract

Tyrosinase involves in the first two key steps in melanogenesis and therefore it has been a target for extensive screening efforts for identification of depigmenting agents for a long time. In vitro cell free tyrosinase assay was carried out to see if *F. deltoidea* extract showed any direct inhibitory effect against the key enzyme in the whole melanogenesis. The result shown in the Fig. 3 clearly demonstrated that marked inhibition of tyrosinase activity was made by treating *F. deltoidea* extract with dose-dependent manner. A positive control, kojic acid showed strong tyrosinase inhibitory effect as expected but arbutin, another positive control, inhibited slightly at the treated concentration. This result suggests that significant reduction of melanin content by treatment of *F. deltoidea* extract (Fig. 2) might be due to at least in part the inhibition of tyrosinase activity in the cells.

Intracellular tyrosinase activity reduced by *Ficus deltoidea*

Reduction of tyrosinase activity in the melanocytes could be achieved either by direct inhibition of tyrosinase itself or by repression of tyrosinase gene expression that leads to lowered amount of the protein level in the cells. In the previous section, we have found that *F. deltoidea* extract had significant inhibitory activity on tyrosinase. The effect of *F. deltoidea* extract on the intracellular tyrosinase activity was analyzed. Instead of using mushroom tyrosinase, cell lysates prepared from the B16F1 cells treated with different concentrations of *F. deltoidea* extract were used as tyrosinase source. The same amount of cell lysates calibrated with respect to protein concentration was applied to oxidation reaction with L-dopa. Upon treatment to α-MSH alone, intracellular tyrosinase activity in the B16F1 cells was increased by twofolds as shown in Fig. 4a. Treatment of 800 μM of kojic acid or 2 mM of arbutin did not reduce the tyrosinase activity. This observation is consistent with the fact that they are known to be direct tyrosinase inhibitors. However, treatment of *F. deltoidea* extract yielded statistically significant reduction of the enzyme activity with dose-dependent manner. At highest concentration of *F. deltoidea* extract (0.1%), the tyrosinase activity decreased by 40%.

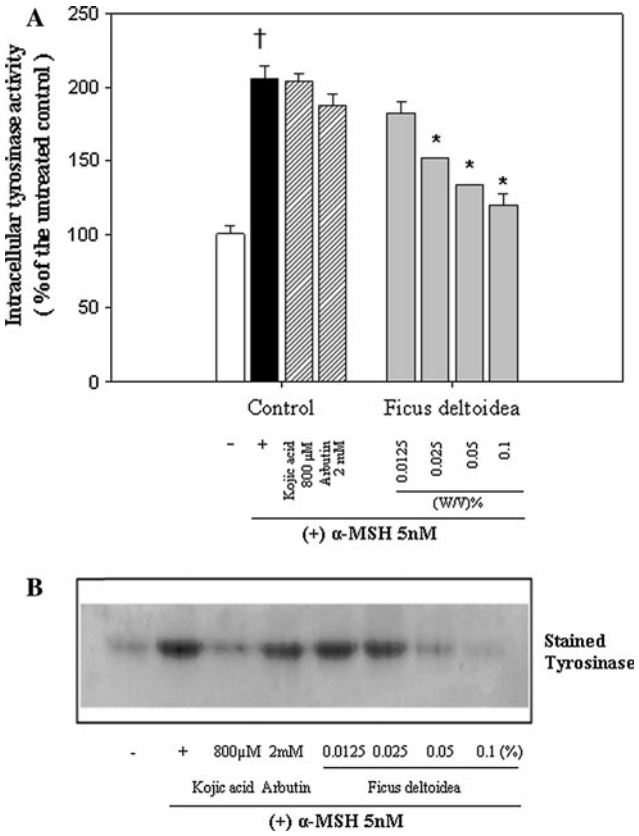

Fig. 4 Intracellular tyrosinase activity of B16F1 melanoma was inactivated by *F. deltoidea.* From the result of intracellular tyrosinase activity using B16F1 melanoma, tyrosinase activity was inhibited by *F. deltoidea* treatment. **a** *F. deltoidea* effectively diminished α-MSH induced intracellular tyrosinase activity. twofold decreased intracellular tyrosinase activity was observed. **b** Decreased tyrosinase activity by *F. deltoidea* was confirmed through the result of tyrosinase zympgraphy. To identufy active form of tyrosinase, tyrosinase zymography was conducted. Figure 4b showed that *F. deltoidea* roles in degradation of α-MSH activated tyrosinase in B16F1 melanoma. At the concentration of 0.1%, almost none of activated tyrosinase was left proved from L-DOPA staining. Results are the average of three independent experiments ± SD. $^*P < 0.01$ compared to (+) α-MSH control

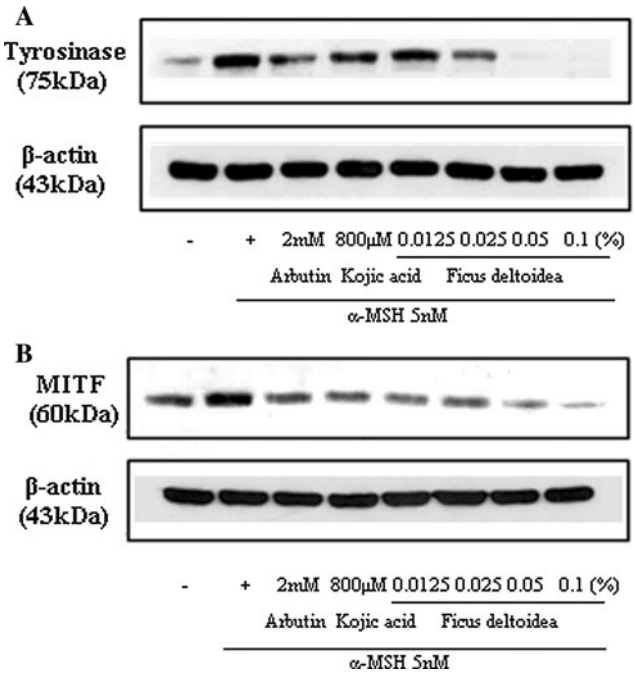

Fig. 5 Tyrosinase and MITF expression detected by immunoblotting. **a** Tyrosinase expression was shown to be lessened through western blot. *β*-actin expression indicated that same amounts of protein were loaded in all lanes. On that condition, tyrosinase expression was induced by treatment of α-MSH and that induction was suppressed by treatment of *F. deltoidea*. The result of Fig. 5a showed that *F. deltoidea* worked as an inhibitor of tyrosinase expression which directly regulates melanin synthesis. **b** MITF expression was shown to be lessened through western blot. *β*-actin expression qualified that same amounts of protein were loaded in all lanes. MITF is a transcription regulator which is related to activation of melanogenic proteins like tyrosinase. From the result of Fig. 5b, *F. deltoidea* down-regulated MITF expression induced by α-MSH treatment

Tyrosinase zymography confirms suppressive effect of *F. deltoidea* extract on the intracellular tyrosinase activity

Tyrosinase activity can also be analyzed after separated by SDS-PAGE. Tyrosinase separation based on the molecular weight on a gel can exhibit its enzymatic activity so that it can oxidize L-dopa solution applied on the gel to form dark-colored DOPA quinone [27]. As shown in Fig. 4b, α-MSH treatment created dark black band compare to that of untreated control. *F. deltoidea* extract treated at concentrations 0.0125, 0.025, 0.05, and 0.1%. Tyrosinase activity was suppressed to the level of α-MSH untreated control at concentration 0.05% and virtually no tyrosinase activity exhibited at concentration 0.1%. The results proved that tyrosinase zymography seemed to be quite-well correlated with the measurement of intracellular tyrosinase activity. The only difference observed was that kojic acid decreased the tyrosinase acitivity, which was unexpected result since it was known as a direct tyrosinase inhibitor.

Immunoblot showed that *F. deltoidea* down-regulated tyrosinase expression

Decreased intracellular tyrosinase activity by *F. deltoidea* extract suggests that the regulation might be exerted at the level of tyrosinase gene expression [15]. In order to confirm this notion, immunoblot analysis for the protein of tyrosinase from the cells treated with different concentrations of *F. deltoidea* extracts was performed using *β*-actin as an internal control. The marked enhancement of 75 kDa tyrosinase upon treated with α-MSH was well correlated with increase of intracellular tyrosinase activity with the same condition. Results shown in Fig. 5a, clearly demonstrated that the amount of tyrosinase protein was dose dependently decreased by the treatment of *F. deltoidea* extract.

F. deltoidea also down-regulated MITF expression

Since MITF is a key transcription factor that modulates the expression of the most melanogenic genes, the effect of *F. deltoidea* extract on the expression of MITF was investigated too. As shown in Fig. 5b, the expression of MITF was also reduced with dose-dependent manner by *F. deltoidea* extract. The results indicated that down-regulation of tyrosinase gene expression by *F. deltoidea* water extract could be due to reduced level of MITF. However, whether this reduction of MITF protein was caused by lowered expression of the MITF gene or degradation of the MITF protein was not determined in this experiment.

Discussion

F. deltoidea, a popular medicinal herb in Malaysia, has been known to have beneficial efficacy for woman. Therefore, it has been orally consumed by local women before and after giving birth as a drinking tea. However, no studies ever conducted with respect to topical applications of *F. deltoidea* either as dermatological agents treating skin diseases or as a functional cosmetic ingredient. We intended in this study to find out new whitening materials since, there are couple of previous reports indicating that it has strong anti-oxidant activity and also contains variety of phytochemicals such as flavanoid, tannins, triterpenoids, proanthocyanins, and phenolics [14]. This study is the second outcome of our recent efforts that aim to investigate Malaysia's traditional medicinal herbs for their potential applications to topical uses. *Labisia pumila*, like *F. deltoidea* is also known to be efficacious against health problems after giving birth. Recently, Choi et al. [4] showed that Labisia pumila showed potent radical-scavenging activity and had strong anti-photoaging efficacy. In our preliminary data found that *F. deltoidea* also seemed to have similar anti-aging activity (unpublished data).

Melanin biosynthesis is the primary cause of pigmentation of human skin and diverse factors such as UV light exposure, inflammatory cytokines, stress and genetic factors are known to be involved in regulation of melanogenesis [9, 20]. When UV is irradiated, melanogenesis is initiated by binding of α-MSH to its authentic receptor MC1R, which leads to increase of cAMP level in the melanocytes [10]. Synthesised melanin is divided into two types, eumelanin and pheomelanin and human skin color is distinguished from types of melanin or mixed rates of them [19]. In accordance with a tendency of increasing demand to consume products related to remedy of pigmental disorders or whitening cosmeceuticals, *F. deltoidea* was evaluated for the possibility of anti-melanogenic efficacy using cultured B16F1 melanoma.

The formation of ROS is stimulated upon UV irradiation and known to be an early event caused by UV in skin. Enhanced amount of ROS probably triggers an inflammatory signaling pathway, which eventually causes downstream cellular activities leading to inflammation, skin aging, and melanogenesis. Therefore, ROS scavenge become a primary target to prevent photoaging and melanin synthesis. Anti-melanogenic activity of ascorbic acid is thought to be due to its ROS scavenging activity. *Ficus* extract is shown to have comparable anti-oxidant activity as a total solid content base suggesting that there may be a compound showing even stronger antioxidant than ascorbic acid.

In the current study, *F. deltoidea* was targeted on a skin pigmentation inhibitor and melanin assay, tyrosinase activity inhibition assay, tyrosinase zymography, immunoblot were conducted to prove. *F. deltoidea* remarkably inhibited α-MSH induced melanin synthesis in cultured B16F1 melanoma and restraint at concentration of 0.1%, was powerful than 800 μM of kojic aicd or 2 mM of arbutin. As reduced melanin resulted from melanin assay showed, melanin synthesis regulated enzyme [21], tyrosinase activity was identified. α–MSH induced intracellular tyrosinase activity using B16F1 melanoma was considerably lessened to twofold.

There are two different modes of action with respect to inhibition of melanin synthesis in melanocytes. One is direct inhibition of tyrosinase enzyme activity and the other is the suppression of tyrosinase gene expression at the transcription level so that the level of tyrosinase protein is reduced in the cells. Many anti-melanogenic agents such as kojic acid, hydroquinone, and arbutin fall in the first category, direct inhibitor of tyrosinase. On the other hand, some agents exert their anti-melanogenic activities by down-regulating the expression of tyrosinase gene, but they do not have any direct inhibitory effect on tyrosinase in general [6]. However, our results clearly showed that *F. deltoidea* extracts have both activities: direct inhibition of mushroom tyrosinase activity in vitro and suppression of genes encoding tyrosinase and its key transcription factor MITF. The results suggest that *F. deltoidea* extract contain at least two distinct active components that differ in their respective mechanism behind the anti-melanogenic effect. Experiments to elucidate relevant signature compounds are underway.

Tyrosinase zymography, a method to identify intracellular tyrosinase existence, proved that intracellular tyrosinase was degraded by *F. deltoidea* up to α-MSH none treated control level. Also, mushroom tyrosinase activity assay resulted that *F. deltoidea* reacts an inhibitor of mushroom tyrosinase oxidation. From these results, we can conclude that the decrement of α-MSH induced melanin synthesis is caused by a combined mechanism that

reduction of intracellular tyrosinase existence with reduction of direct tyrosinase catalysis process. To verify intracellular tyrosinase regulation, MITF, a regulating factor at transcription level during pigmentation, expression was conducted by western blot [1]. MITF expression was decreased by *F. deltoidea* more than α-MSH none treat control at concentrations 0.05 and 0.1%. In a conclusion, *F. deltoidea* has strong possibilities being whitening cosmetic materials by down-regulation of cellular melanogenic components like tyrosinase, MITF and direct catalytic process as well.

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