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Inhibitory effect of a water extract from *Pemphis acidula* on melanogenesis in mouse B16 melanoma cells

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Synopsis

The inhibitory effect of a water extract from *Pemphis acidula* on melanogenesis in mouse B16 melanoma cells was investigated. The results showed that the *P. acidula* extract (PAE) inhibited melanogenesis in 3-isobutyl-1-methylxanthin (IBMX)-stimulated B16 cells in a dose-dependent manner, with an IC_{50} value of 33.5 μg/ml. In addition, PAE also inhibited cellular tyrosinase activity. Moreover, western blot and real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analyses respectively confirmed that PAE down-regulated levels of tyrosinase protein and its mRNA in IBMX-stimulated B16 cells. These results demonstrated that PAE inhibits melanogenesis of B16 cells by reducing tyrosinase gene expression. From the present study, PAE is proven to be a good candidate as a skin-whitening agent for treatment of skin hyperpigmentation.

INTRODUCTION

Skin pigmentation is produced by dermal melanocytes. Melanogenesis has been defined as the entire process leading to the formation of dark macromolecular pigments, i.e., melanin, which are formed by a combination of enzymatically catalyzed and chemical reactions (1). Melanogenesis is initiated in melanosomes, the special organelles of melanocytes, with the first step of L-tyrosine oxidation to L-dopa (L-3,4-dihydroxyphenylalanine) and then to dopaquinone, which is catalyzed by tyrosinase. This is a rate-limiting step in melanin synthesis because the remainder of the reaction sequence can proceed spontaneously at a physiological pH value. Although melanin mainly plays a photoprotective role, the accumulation of abnormal amounts of melanin in different parts of the skin, which results in pigmented patches of skin, might become an esthetic problem.

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Therefore, several studies have focused on the inhibition of tyrosinase activity and the prevention of abnormal pigmentation (2,3).

A previous study has reported that some cytokines and growth factors play important regulatory roles in melanogenesis (4). α-Melanocyte stimulating hormone (α-MSH) is the most well-studied hormone. This hormone binds to its receptor, melanocortin receptor 1 (MC1R), on the membrane of melanocytes and stimulates melanogenesis via the GPCR (G protein-coupled receptor)-cAMP-MITF (microphthalmia-associated transcription factor) pathway where the melanogenesis-related enzymes, including tyrosinase and tyrosinase-related proteins 1 and 2 (TRP1 and TRP2) are up-regulated. In addition to α-MSH, other intracellular cAMP elevation agents such as forskolin or 3-isobuty1-1-methylxanthin (IBMX) also stimulate melanogenesis through the same signal pathway as does α-MSH. Accordingly, agents blocking the signal pathway would exhibit depigmentation against melanocytes (5,6).

In the present study, we screened more than 200 crude extracts of traditional Chinese medicinal herbs to identify their applicability as skin-lightening agents. The *P. acidula* extract (PAE) was found to have strong inhibitory activity on melanogenesis in mouse B16 melanoma cells. The inhibitory effect of the extract on melanogenesis of the cells was investigated in advance.

MATERIALS AND METHODS

PREPARATION OF PAE

The dried powder of the bark (235.0 g) of *P. acidula* was extracted with one liter of water at room temperature overnight four times, followed by filtration at the end of each extraction. The flow-through extracts were concentrated in a vacuum and combined to yield a black syrup (14.3 g).

CHEMICALS AND ANTIBODIES

Arbutin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton X-100, phenylmethylsulfonyl fluoride (PMSF), L-dopa, dimethyl sulfoxide (DMSO), trypsin/EDTA, synthetic melanin, and IBMX were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-tyrosinase antibodies (#62914) and protease inhibitor cocktail were obtained from Abcam (Cambridge, MA). Anti-β-actin antibodies (#3662) were purchased from Bio Vision Inc. (Irvine, CA). All other chemicals were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan) and were of analytic reagent grade.

CELL CULTURES AND DRUG TREATMENTS

Mouse B16 melanoma cells (4A5) were obtained from the Bioresources Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37°C in a humidified, CO_2-controlled

(5%) incubator. The cells were seeded at an appropriate cell density in a 24-well or a six-well plate. After 1 d of incubation, the cells were treated with various concentrations of the drugs in the absence or presence of a stimulation agent (100 μM of IBMX) for another 6 h (qRT-PCR), 16 h (tyrosinase activity assay and western blot), or 48 h (melanin determination). Thereafter, the cells were harvested and used for various assays.

MEASUREMENTS OF CELL VIABILITY

An MTT assay was performed to examine the viability of cells. After the cells were incubated with the samples for 48 h, the culture medium was removed and replaced with 1 mg/ml of MTT solution dissolved in phosphate-buffered saline (PBS) and incubated for an additional 2 h. The MTT solution was then removed and DMSO was added, following which the absorbance of the dissolved formazan crystals was determined at 570 nm by a spectrophotometer.

DETERMINATION OF MELANIN CONTENT

At the end of cell cultivation, the cells were harvested and washed twice with PBS. The pelleted cells were homogenized in lysis buffer containing 20 mM of sodium phosphate (pH 6.8) and 1% Triton X-100 at 4°C with 30 strokes in a Dounce homogenizer. After centrifugation at 15,000*g* for 15 min, the melanin pellets were dissolved in 1 N NaOH containing 20% DMSO for 1 h at 95°C. The absorbance at 490 nm was measured, and the melanin content was measured using the authentic standard of synthetic melanin.

MEASUREMENT OF CELLULAR TYROSINASE ACTIVITY

To determine the tyrosinase activity in the crude extract, a source of crude cellular tyrosinase was obtained by homogenizing drug-treated or untreated cells in 20 mM of sodium phosphate (pH 6.8), 1% Triton X-100, and 1 mM of PMSF at 4°C with 30 repeated strokes in a Dounce homogenizer. Detergent was used to release the membrane-bound tyrosinase from the melanosomes. The lysates were centrifuged at 15,000 rpm for 15 min to obtain the supernatant as the source of crude cellular tyrosinase. The protein content in the supernatant was determined using a Bradford assay with BSA as the protein standard. Tyrosinase activity was then determined as follows: 1 ml of the reaction mixture contained 50 mM of phosphate buffer (pH 6.8), 2.5 mM of L-dopa, and 500 μg of the supernatant protein, and was incubated at 37°C for 15 min, following which the dopachrome formation was monitored by measuring absorbance at a wavelength of 475 nm.

WESTERN BLOT ANALYSIS

The cells were washed three times in ice-cold PBS, and lysed in cold lysis buffer (20 mM of sodium phosphate (pH 6.8), 1% Triton X-100, 1 mM of PMSF, and 1 mM of EDTA) containing protease inhibitor cocktail (Abcam, Cambridge, UK). An aliquot of the lysate

was used to determine the protein content with a Bradford assay, using BSA as the standard. The proteins (100 μg) were separated using 10% SDS-polyacrylamide gel electrophoresis and blotted onto polyvinyl difluoride (PVDF) membranes (MP Biomedicals Co., Irvine, CA). The membranes were blocked with 5% non-fat skim milk in TBS-T buffer. Tyrosinase and β-actin (as an internal control) were detected using rabbit polyclonal antibodies and mouse monoclonal anti-β-actin antibodies, respectively. The membranes were further incubated with horseradish peroxidase-conjugated secondary antibody. All bound antibodies were then detected using an Amersham ECL system (Amersham Pharmacia Biotech, Piscataway, NJ). The signal intensity of each band was quantified with a GS-700 densitometer system (Bio-Rad, CA) equipped with an integrator, and normalized with that of the internal control.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (qRT-PCR)

A quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on the ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA) using Fast SYBR® Green Master Mix (Applied Biosystems). Total RNA was extracted using an RNeasy® mini Kit (Qiagen, CA) according to the manufacturer's instructions. The quality of the total RNA sample was evaluated by determining the OD_{260}/OD_{280} ratio. To prepare a cDNA pool from each RNA sample, total RNA (2 μg) was reverse transcribed at 42°C for 90 min in the presence of oligo(dT) primers (MD Bio. Co., Taipei, Taiwan) and reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany). The oligonucleotide primers for mouse tyrosinase (forward, 5′-GGCCAGCTTTCAGGCAGAGGT-3′; reverse, 5′-TGGTGCTTCATGGGCAAAATC-3′) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (forward, 5′-ACCACAGTCCATGCCATCAC-3′; reverse, 5′-TCCACCACCCTGTTGCTGTA-3′) were used. After the initial incubation of 2 min at 50°C, the cDNA was denatured at 95°C for 10 min followed by 40 cycles of PCR (95°C, 15 s, 60°C, 60 s). All results were obtained from at least three independent experiments. The mRNA level of tyrosinase was normalized using GAPDH as an internal control.

STATISTICAL ANALYSIS

All the data in the present study were obtained as averages of experiments that were performed in triplicate and are expressed as means ± S.D. Statistical analysis was performed by the Student's *t*-test. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

EFFECT OF PAE ON CELL VIABILITY

Safety is an important criterion for a skin-lightening drug. Therefore, before investigating the effect of PAE on the melanogenesis of mouse B16 melanoma cells, the concentration range of the extract that is nontoxic to the cells should be determined. The viability of drug-treated cells was determined by the MTT method. Arbutin was used as the

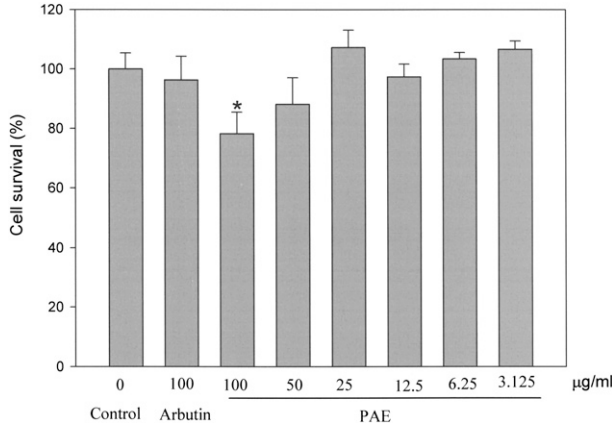

Figure 1. Effect of PAE on B16 cell viability. The cells were seeded in 24-well plates for 1 d and then treated with various dosages of PAE (100–3.125 mg/ml) for 2 d. The cell viability was then examined by the MTT assay, as described in the Experimental section.

control in the present study. As shown in Figure 1, the result indicated that 50 μg/ml PAE does not exert significant cytotoxicity to mouse B16 melanoma cells. In order to avoid the cytotoxic effect of PAE, we used a PAE concentration of up to 50 μg/ml in the depigmenting experiments of the present study.

EFFECT OF PAE ON MELANOGENESIS AND TYROSINASE ACTIVITY IN B16 CELLS

To study the anti-melanogenic effect of PAE in B16 cells, the melanin content of the cells in each treatment was determined. The results are presented in Figure 2. The melanin content of B16 cells increased considerably after stimulation by IBMX. Thereafter, PAE

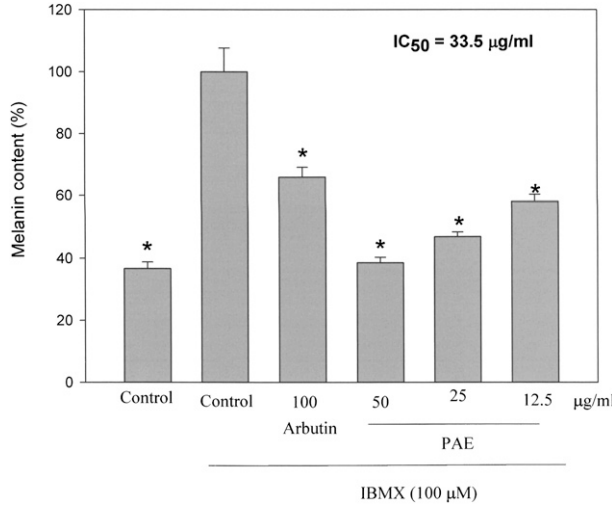

Figure 2. Effect of PAE on melanogenesis in B16 cells. The cells were cultivated for 1 d and then stimulated with 100 μM of IBMX for 2 d in the absence or presence of PAE. The melanin content of the cells was determined using spectrometry, as described in the Experimental section.

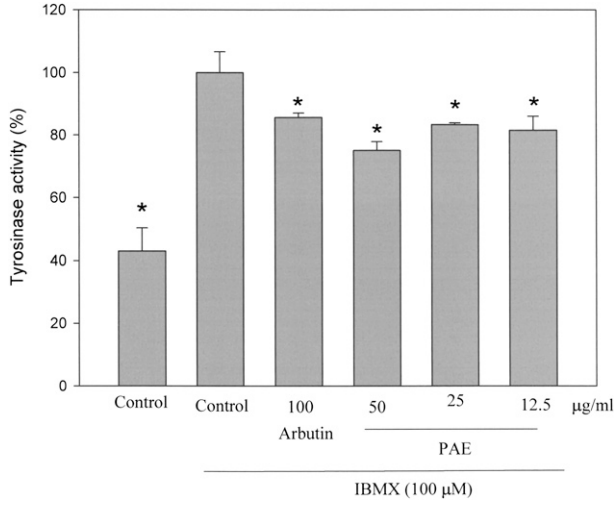

Figure 3. Effect of PAE on cellular tyrosinase activity of B16 cells. The cells were cultivated for 1 d and stimulated with 100 µM of IBMX in the absence or presence of various dosages of PAE or arbutin for 2 d. Cellular tyrosinase activity in the cells was determined using spectrometry as described in the Experimental section.

treatment resulted in a significant decrease in the melanin content of IBMX-stimulated B16 cells. In addition, the melanogenic inhibition of PAE showed a dose-dependent manner with an IC_{50} value of 33.5 µg/ml. When the inhibitory potency of PAE and arbutin were compared, PAE exhibited a stronger activity than that of arbutin.

Because tyrosinase plays an important role in melanogenesis, we determined the effect of PAE on tyrosinase activity. After PAE treatments, B16 cells were lysed to obtain cellular tyrosinase. We measured the enzyme activity by using L-dopa as an enzyme substrate. The result is shown in Figure 3. The cellular tyrosinase activity was significantly increased after stimulation by IBMX, while PAE treatments would significantly inhibit the stimulated cellular tyrosinase activity. Hence, the result suggested that PAE reduced melanogenesis of IBMX-stimulated B16 cells via down-regulated cellular tyrosinase activity.

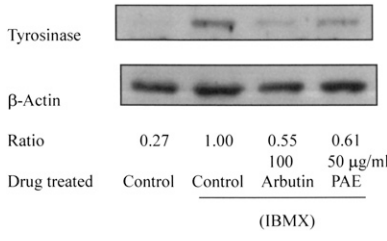

Figure 4. Effect of PAE on amounts of tyrosinase protein. Cells were inoculated in 24-well plates for 1 d and then stimulated by 100 µM of IBMX with or without the test drug. The cells were harvested and the total protein was analyzed by western blot as described in the Experimental section. The band intensity of tyrosinase was normalized by that of β-actin, and the normalized band intensity in the IBMX-stimulated control was recalculated to be 1.

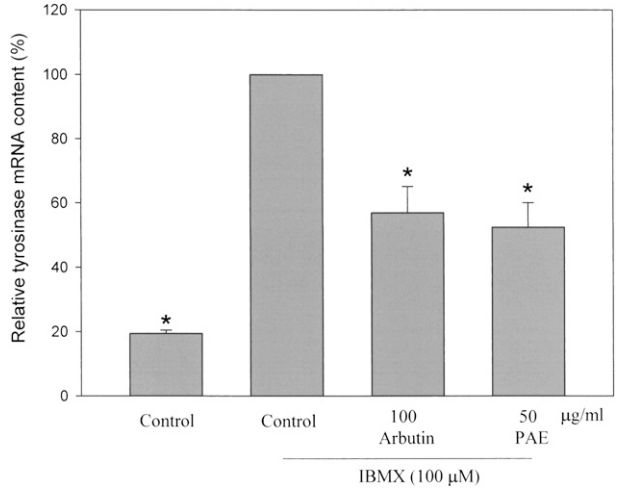

Figure 5. Effect of PAE on the expression of the tyrosinase gene. B16 cells were seeded in six-well plates for 1 d and treated with or without 100 μM of IBMX with or without the test drug for 6 h after which total RNA isolation and then reverse transcription was carried out to obtain cDNAs that were used for quantitative real-time PCR. Relative mRNA expression was calculated by the ΔΔCt method, where ΔCt is the value obtained by subtracting the Ct value of GAPDH mRNA from the Ct value of the tyrosinase mRNA and ΔΔCt is the value obtained by subtracting the ΔCt value of a reaction from the ΔCt value of the control with IBMX stimulation. Specifically, relative mRNA expression is expressed as $2^{-\Delta\Delta Ct}$. Results represent the mean ± SD of three independent experiments. *Statistically significant ($p < 0.05$) difference between the control and the treated cells.

EFFECT OF PAE ON TYROSINASE PROTEIN AND ITS mRNA LEVELS IN B16 CELLS

To study the inhibitory effect of PAE on melanogenesis in more detail, we conducted western blot and qRT-PCR analyses to verify levels of tyrosinase protein and its mRNA in the PAE-treated cells. The results are shown in Figure 4 (western blot) and Figure 5 (qRT-PCR). After stimulation by IBMX, both tyrosinase protein (Figure 4) and its mRNA (Figure 5) were significantly increased. Moreover, the increased levels of both tyrosinase protein and its mRNA in the IBMX-stimulated cells were down-regulated by PAE treatments. These results reasonably explain the previous finding wherein both cellular tyrosinase activity (Figure 3) and melanin content (Figure 2) were decreased in the PAE-treated cells due to the inhibitory effect on tyrosinase gene expression by the extract in the IBMX-stimulated mouse melanoma B16 cells.

CONCLUSION

Our results clearly demonstrate that PAE is an effective melanogenesis inhibitor that functions via down-regulation of tyrosinase expression. These results indicate that PAE may be useful in the treatment of skin hyperpigmentation.

ACKNOWLEDGMENTS

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