Effects of wogonin on the mechanism of melanin synthesis in A375 cells

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Abstract. The present study aimed to investigate the effect of wogonin on the mechanism of melanin synthesis in the A375 melanoma cell line. A375 cells, cultured *in vitro,* were treated with wogonin and the activity of tyrosinase (TYR) and melanin synthesis were examined via MTT assay, L-dopa oxidation assay and an NaOH lysis assay. Protein expression levels of TYR and c-Jun N-terminal kinase (JNK) were examined via western blotting. mRNA expression levels of TYR, tyrosinase related protein (TRP)-1, TRP-2, extracellular signal-regulated kinase (ERK)-1, ERK-2 and JNK-2 were analyzed by reverse transcription-quantitative polymerase chain reaction. Furthermore, the effect of wogonin on estrogen receptor inhibitor (ICI182780) and ERK pathway inhibitor (U0126) was investigated. Safe doses of wogonin (10, 1, 10^{-1}, 10^{-2} or 10^{-3} μmol/l) significantly inhibited melanin synthesis and TYR activity ($P<0.05$). Wogonin (10 μmol/l) inhibited the protein expression levels of TYR, JNK and mRNA expression levels of TYR, TRP-1, TRP-2, ERK-1, ERK-2, JNK-2 in A375 cells ($P<0.01$). The estrogen receptor inhibitor, ICI182780, and MEK inhibitor, U0126, significantly reversed the effects of wogonin on protein and mRNA expression levels of TYR, TRP-1, TRP-2, ERK-1, ERK-2 and JNK-2 (all $P<0.01$). To conclude, the present study identified that wogonin is able to inhibit the synthesis of melanin in A375 cells, through inhibiting protein and mRNA expression levels of TYR, TRP-1, and TRP-2, and ERK1, ERK2 and JNK2, respectively.

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Key words: wogonin, melanin, estrogen receptor-mitogen activated protein kinase, A375 cells

Introduction

Wogonin is a flavonoid-like chemical compound that was identified in *Eucommia ulmoides* (1). *Eucommia ulmoides* extract is a valuable Chinese herbal medicine which is considered a raw material of gum as it is sweet, has a pungent taste and is non-toxic. Furthermore, this compound may aid hepatic and renal function, improve memory and relieve fatigue (2). Previous studies have demonstrated that *Eucommia ulmoides* possesses estrogen-like effects (3,4). The extract of *Eucommia ulmoides* is able to decrease the bone loss in ovariectomized rats with diabetes mellitus through increasing levels of estrogen (3). Phytoestrogen properties of *Eucommia ulmoides* include: Flavonoid components (wogonin and baicalein), lignan components (pinoresinol and diglucoside) and iridoid glycosides (aucubin, geniposidic acid and eucommiol), which have the material basis to produce estrogen-like effects (5,6). Phytoestrogen, a bisphenol structure chemical compound, elicits estrogen-like effects without provoking estrogen-like side effects such as increased risk of breast cancer, endometrial cancer and ovarian cancers (7), and is an area of interest for the treatment of skin disease as a substitute for estrogen (8). A previous study revealed that *Eucommia ulmoides* may inhibit melanocyte proliferation, tyrosinase activity and melanin synthesis (9). Betulinic acid, a phytoestrogen identified in *Eucommia ulmoides*, may inhibit the proliferation of melanocyte and melanoma cells (10) and was revealed to induce cell apoptosis and the expression of induced myeloid leukemia cell differentiation protein.

The present study investigated the effect of wogonin on the mechanism of melanin synthesis in A375 cells. The effect of wogonin on melanogenesis may be associated with the regulation of the ER-MAPK signaling pathway and TYR activity.

Materials and methods

Cells and reagents. Human A375 cells were supplied by the Cell Center of Chinese Academy of Sciences (Beijing, China). Tissue and cell lysis solution histiocyte lysate (WIP; Beyotime Institute of Biotechnology, Haimen, China); MTT (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany);

primer (Shanghai Shengong Biology Engineering Technology Service, Ltd., Shanghai, China); Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA); goat anti-mouse immunoglobulin G (IgG) marked by horseradish peroxidase (HRP; cat no. BST08k13B; Wuhan Boster Biological Technology, Ltd., Wuhan, China); mouse anti-β-actin monoclonal antibody (cat no. 140925; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing China); mouse anti-tyrosinase monoclonal antibody (cat no. F0811; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); and mouse anti-c-Jun N-terminal kinase (JNK) monoclonal antibody (cat no. G0913) and TRIzol reagent (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Instruments. Nano-100 Micro spectrophotometer (Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, China); TCL6G-C table-top high-speed refrigerated centrifuge (Shanghai Anting Scientific Instrument Factory, Shanghai, China); TProfessional Basic PCR Amplification instrument (Biometra GmbH, Göttingen, Germany); MK3 enzyme microplate reader (Redian, Shanghai, China); DYCZ-40B transfer electrophoresis (Beijing Liuyi Biotechnology Co., Ltd., Beijing, China); and SmartChemi500 one piece micro-ChemiDoc XRS gel imaging system (Beijing Sage Creation Science Co., Ltd., Beijing, China).

Drugs. Wogonin with a purity >98% (cat no. MUST-13092303; Nanjing Zelang Medical Technology Co., Ltd., Nanjing, China); estradiol with a purity >98% (cat no. L750N46; J&K Scientific, Ltd., Beijing, China); ICI182780 (cat no. 20A/129473; Tocris Bioscience, Bristol, UK); and U0126 (S110202; Selleck Chemicals, Houston, TX, USA).

Preparation of drug solution. A total of 5.5 mg estradiol was dissolved in 4 ml anhydrous alcohol and combined with DMEM to prepare a stock solution at a concentration of 20 μmol/l, which was diluted to $1x10^{-3}$ μmol/l when used. Wogonin (5.69 mg) was dissolved in DMEM to prepare a solution with an initial concentration of 200 μmol/l, diluted to 10 μmol/l when used. ICI182780 (5 mg) was dissolved in 1% dimethyl sulphoxide (DMSO), and added to DMEM to prepare a solution with an initial concentration of 200 μmol/l, which was diluted to 1 μmol/l when used. U0126 (8.53 mg) was dissolved in 1% DMSO and combined with DMEM to prepare a stock solution at a concentration of 400 μmol/l, which was diluted to 10 μmol/l when used. All solutions were filtered and sterilized using a 0.22-μm filter membrane, and stored at -20˚C.

Cell grouping and culture. A375 cells were grown to 80% confluence and digested with 0.25% Trypsin, centrifuged at 1,000 x g for 5 min at room temperature, and resuspended. Subsequently, cells were transferred onto 96-well plates (200 μl DMEM with ~4,500 cells/well) or 6-well plates (2 ml DMEM with $24x10^4$ cells/well) and incubated at 37˚C in an atmosphere containing 5% CO_2 for 24 h. The medium was discarded and cells were divided into different groups according to the treatment administered, including: The control group, cells in the presence of DMEM without treatment; estradiol group, cells in the presence of estradiol (10^{-3} μmol/l); wogonin group, cells in the presence of wogonin (10 μmol/l) solution; wogonin + ICI182780 group, cells in the presence of ICI182780 (1 μmol/l) and 10 μmol/l of wogonin; and wogonin + U0126 group, cells in the presence of U0126 (10 μmol/l) and 10 μmol/l of wogonin. All groups were cultured for 48 h. There are 6 wells for duplicate in 96-well plates, 4 wells for duplicate in 6-well plates.

Cell activity. Cells were seeded at a density of $8x10^3$ cells/well. MTT solution (20 μl) was added into each well of the 96-well plates and cultured for 4 h at 37˚C. Following the discarding of the solution, 150 μl of DMSO solution was added to dissolve the crystals and incubated at 37˚C using an incubator shaker for 10 min. To detect the optical density (OD), plates were read at a wavelength of 490 nm using a microplate reader. The same volume of PBS was used as a blank control.

Melanin synthesis. Solution in 6-well plates was discarded and cells were washed with phosphate-buffered saline (PBS) twice, digested with 1 ml 0.25% pancreatin for 1 min, and 1 ml of DMEM was subsequently added to terminate digestion. Cell suspension was collected in 15 ml centrifuge tubes, and centrifuged at 1,000 x g for 5 min at 25˚C and the supernatant was discarded. A total of 100 μl of 1 mol/l NaOH was added and the solution was mixed and incubated at 37˚C for 1 h. Following this, 400 μl of double distilled water was added and 100 μl of solution from each centrifuge tube was transferred onto 96-well plates. To detect the OD, plates were read at a wavelength of 490 nm using a microplate reader.

TYR activity. Solution was discarded and cells in the 96-well plates were washed with PBS twice, 100 μl Triton 1% was added in each well, and cells were frozen at -80˚C for 30 min before 50 μl PBS (supplemented with 0.2% L-dopa; pH 6.8) was added and cells were incubated at 37˚C for 3 h. To detect the OD, plates were read at a wavelength of 490 nm using a microplate reader.

Western blot analysis. To analyze the protein expression of TYR and JNK, total protein of each group in 6-well plates were separated by SDS-PAGE. When cells reached 80% confluence, they were incubated with WIP cell lysis buffer for 30 min at 0˚C and centrifuged at 30,000 x g for 15 min at 4˚C to extract the total protein. A total of 30 μg total protein was loaded into each lane and separated by 12% SDS-PAGE. The proteins were transferred onto a polyvinylidene difluoride membrane, and blocked with 5% skim milk for 1 h at room temperature to prevent non-specific binding. The membrane was subsequently incubated with primary antibodies (all 1:300) overnight at 4˚C. The primary antibodies used were as follows: Mouse anti-β-actin monoclonal antibody, mouse anti-tyrosinase monoclonal antibody and mouse anti-c-Jun N-terminal kinase (JNK) monoclonal antibody. The membrane was washed with TBST 3 times for 10 min and incubated with the corresponding HRP goat anti-mouse IgG (1:10,000) for 1 h at room temperature. The membrane was washed again and bands were visualized using enhanced chemiluminescence. Images were captured and analyzed. The quantity of target protein was indicated by the ratio of its gray value to an internal reference (β-actin). Bands were quantified using lane

Table I. Primer sequences.

Gene	Upstream primer	Downstream primer	Amplified fragment (bp)
TYR	5'TCACGGCTCTGTTGAATGTCT3'	5'CTGAAGTTGGGCGAGATGAT3'	300
TRP-1	5'ACATCATTCCCTCACCAAAGAC3'	5'AGAAGTCCGAAAGCCAAGTAAA3'	303
TRP-2	5'GTTCCTTTCTTCCCTCCAGTG3'	5'TTCCTTTATTGTCAGCGTCAG3'	300
ERK1	5'GGGAGGTGGAGATGGTGAAG3'	5'AGCAGGTTGGAGGGCTTTAGAT3'	441
ERK2	5'ACCCACACAAGAGGATTGAAGT3	5'AAAAGCCACAACTACCAGAAAC3'	353
JNK2	5'CCTTCTTTACCAGATGCTTTGTG3'	5'ATACGGTCAGTGCCTTGGAATA3'	303
β-actin	5'CGTGGACATCCGCAAAGAC3'	5'AAGAAAGGGTGTAACGCAACTA3'	302

TYR, tyrosinase; TRP-1, tyrosinase related protein 1; TRP-2, tyrosinase related protein 2; ERK1, extracellular signal-regulated kinase 1; ERK2, extracellular signal-regulated kinase 2; JNK2, c-Jun N-terminal kinase 2.

Table II. Effects of wogonin on A375 cell activity.

Group	Concentration (μmol/l)	OD	Cell proliferation rate (%)
Control group	-	0.470±0.014	100.00
Estradiol group	10^{-3}	0.570±0.023[a]	121.70[a]
Wogonin group	10	0.448±0.032	95.32

Data are presented as the mean ± standard deviation (n=6). [a]P<0.01 vs. the control group. OD, optical density.

1D gel imaging analysis software (SmartChemi 500; Beijing Innovation Technology, Ltd., Beijing, China).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. In order to detect the expression levels of TYR, TRP-1, TRP-2, extracellular signal-regulated kinase (ERK)1, ERK2 and JNK2 mRNA, total RNA was extracted from each group of cells in 6-well plates using TRIzol reagent. The concentration and purity of the total RNA extracted from A375 cells were measured using the Nano-100 micro-spectrophotometer. A total of 3 μg of RNA was reverse transcribed into cDNA using the AMV First-Strand cDNA Synthesis kit (Sangon Biotech Co., Ltd., Shanghai, China). Total RNA was combined with 1 μl oligo(dT) primer and made up to 11 μl with RNase-free water and centrifuged at 5,000 x g for 3-5 sec at 25˚C. The centrifuge tube was placed in a water bath at 70˚C for 5 min followed by an ice bath for 30 sec. Primer sequences were designed and provided by Sangon Biotech Co., Ltd. and are indicated in Table I, along with the β-actin internal reference. A final reaction volume of 25 μl was used for PCR amplification, comprising 16.1 μl ddH_2O, 2.5 μl 10X PCR buffer, 2.5 μl dNTP, 1.3 μl $MgCl_2$,1.5 μl cDNA, 0.5 μl forward primer, 0.5 μl reverse primer and 0.1 μl Taq DNA polymerase. cDNA amplification conditions included: Initial denaturation at 94˚C for 2 min; denaturation for 30 sec at 94˚C; annealing for 40 sec (TYR at 60˚C, TRP-1 at 58˚C, TYP-2 at 58˚C, ERK-1 at 58˚C, ERK-2 at 55˚C, JNK-2 at 55˚C, and β-actin at 55˚C); and extension for 40 sec at 70˚C. A total of 35 PCR cycles were used for the amplification of the samples. A total of 5 μl of PCR amplification products were combined with 1 μl DNA loading buffer (6X) and the PCR products were fractionated using 1.5% agarose gel electrophoresis containing 0.5 μg/ml ethidium bromide at a constant voltage of 120 V for 30 min. Results were detected using the gel imaging system, and analyzed using gel-pro analysis software (SmartChemi 500; Beijing Innovation Technology, Ltd.). mRNA levels of each group were expressed according to the relative value (TYR/β-actin, TRP-1/β-actin, TRP-2/β-actin, ERK-1/β-actin, ERK-2/β-actin, and JNK-2/β-actin).

Statistical analysis. All data were expressed as the mean ± standard deviation. One-way analysis of variance was used to analyze data, using the SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA). A 2:2 comparison was made using Fisher's least significant difference. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of wogonin on cell activity. Effects of wogonin on A375 cell activity are displayed in Table II. Results from the wogonin group indicate that 10 μmol/l of wogonin had no significant effect on A375 cell activity when compared with the control group; therefore, 10 μmol/l of wogonin was used in subsequent experiments.

Effects of wogonin on melanin synthesis of A375 cells. Wogonin was demonstrated to significantly inhibit melanin synthesis, which was indicated by the significant decrease exhibited in the wogonin group (45.99%) when compared with the control group (100%; P<0.01; Table III). Both ICI182780 and U0126 treatment was indicated to reverse the melanin decline elicited

Table III. Effects of wogonin on the melanin synthesis of A375 cells.

Group (μmol/l)	OD	OD treatment group/OD control group (%)
Control group (-)	0.138±0.009	100.00
Estradiol group (10^{-3})	0.167±0.006[a]	121.90[a]
Wogonin group (10)	0.063±0.013[a]	45.99[a]
Wogonin group (10) + ICI182780 (1)	0.106±0.003[a,b]	77.37[a,b]
Wogonin group (10) + U0126 (10)	0.085±0.005[a,b]	62.04[a,b]

Data are presented as the (mean ± standard deviation, n=4), [a]P<0.01 vs. the control group; [b]P<0.01 vs. the wogonin group. ICI182780, estrogen inhibitor; U0126, extracellular signal-regulated kinase inhibitor; OD, optical density.

Table IV. Effects of wogonin on the TYR activity of A375 cells.

Group (μmol/l)	OD	OD treatment group/OD control group (%)
Control group (-)	0.085±0.005	100.00
Estradiol group (10^{-3})	0.106±0.004[a]	124.71[a]
Wogonin group (10)	0.054±0.004[a]	63.53[a]
Wogonin group (10) + ICI182780 (1)	0.071±0.011[a,b]	83.53[a,b]
Wogonin group (10) + U0126 (10)	0.066±0.009[a,b]	77.65[a,b]

Data are presented as the mean ± standard deviation (n=6). [a]P<0.01 vs. the control group; [b]P<0.01 vs. the wogonin group. ICI182780, estrogen inhibitor; U0126, extracellular signal-regulated kinase inhibitor; OD, optical density; TYR, tyrosinase.

Table V. Expression levels of TYR and JNK protein in different groups.

Group (μmol/l)	TYR/β-actin	JNK/β-actin
Control group (-)	0.488±0.018	0.428±0.009
Estradiol group (10^{-3})	0.645±0.020[a]	0.520±0.012[a]
Wogonin group (10)	0.302±0.013[a]	0.214±0.023[a]
Wogonin group (10) + ICI182780 (1)	0.413±0.052[a,b]	0.339±0.035[a,b]

Data are presented as the mean ± standard deviation (n=4). [a]P<0.01 vs. the control group; [b]P<0.01 vs. the wogonin group. ICI182780, estrogen inhibitor; U0126, extracellular signal-regulated kinase inhibitor; OD, optical density; TYR, tyrosinase; JNK, c-Jun N-terminal kinase.

by wogonin, as demonstrated by the significant increase exhibited by the wogonin + ICI182780 and wogonin + U0126 groups (77.37 and 62.04%, respectively) when compared with the wogonin group (45.99%; P<0.01; Table III).

Effects of wogonin on the TYR activity of A375 cells. Wogonin significantly inhibited TYR activity, which was indicated by the significant decrease exhibited by the wogonin group (65.53%) when compared with the control group (100%; P<0.01; Table IV). ICI182780 and U0126 were demonstrated to reverse the decline of TYR activity elicited by wogonin, as indicated by the significant increases observed in the wogonin + ICI182780 group (83.53%) and the wogonin + U0126 group (77.65%) when compared with the wogonin group (63.53%; P<0.01; Table IV).

Effects of wogonin on expression of TYR and JNK protein. The wogonin group indicated that the presence of wogonin significantly inhibited the expression levels of TYR and JNK protein when compared with the control group (P<0.01; Table V and Fig. 1). ICI182780 was indicated to reverse the decline exhibited in the protein expression levels of TYR and JNK initiated by wogonin, as suggested by the significant increase displayed by the wogonin + ICI182780 group (TYR, 0.413±0.052 and JNK, 0.339±0.035) when compared with the wogonin group (TYR, 0.302±0.013 and JNK, 0.214±0.023; P<0.01; Table V).

Effects of wogonin and ICI182780 on the mRNA expression levels of TYR, TRP-1, TRP-2, ERK1, ERK2 and JNK2 in A375 cells. The significantly decreased levels of mRNA expression of TYR, TRP-1, TRP-2, ERK1, ERK2 and JNK2 in the wogonin group suggested that wogonin significantly inhibited the mRNA expression levels of TYR, TRP-1, TRP-2, ERK1, ERK2 and JNK2 when compared with the control group

Table VI. Polymerase chain reaction results of the mRNA expression levels of TYR, TRP-1, TRP-2, ERK1, ERK2, JNK2 mRNA in different groups.

mRNA	Control group	Estradiol group (10^{-3} μmol/l)	Wogonin group (10 μmol/l)	Wogonin group (10 μmol/l)+ ICI182780 group (10 μmol/l)
TYR/β-actin	0.505±0.012	0.666±0.031[a]	0.381±0.019[a]	0.453±0.033[a,b]
TRP-1/β-actin	0.441±0.024	0.515±0.005[a]	0.298±0.012[a]	0.389±0.018[a,b]
TRP-2/β-actin	0.419±0.022	0.584±0.023[a]	0.231±0.012[a]	0.323±0.014[a,c]
ERK1/β-actin	0.501±0.018	0.623±0.021[a]	0.252±0.032[a]	0.453±0.016[a,c]
ERK2/β-actin	0.486±0.019	0.633±0.030[a]	0.292±0.031[a]	0.399±0.022[a,c]
JNK2/β-actin	0.408±0.014	0.513±0.028[a]	0.252±0.021[a]	0.297±0.021[a,c]

Data are presented as the mean ± standard deviation (n=4). [a]P<0.01 vs. the control group; [b]P<005 and [c]P<0.01 vs. the wogonin group. ICI182780, estrogen inhibitor; U0126, extracellular signal-regulated kinase inhibitor; TYR, tyrosinase; TRP-1, tyrosinase related protein 1; TRP-2, tyrosinase related protein 2; ERK1, extracellular signal-regulated kinase 1; ERK2, extracellular signal-regulated kinase 2; JNK2, c-Jun N-terminal kinase 2.

Table VII. Polymerase chain reaction results of the mRNA expression levels of TYR, TRP-1, TRP-2 mRNA in different groups.

mRNA	Control group	Estradiol group (10^{-3} μmol/l)	Wogonin group (10 μmol/l)	Wogonin group (10 μmol/l)+ ICI182780 group (10 μmol/l)
TYR/β-actin	0.576±0.018	0.654±0.040[a]	0.405±0.018[a]	0.511±0.030[a,b]
TRP-1/β-actin	0.462±0.013	0.578±0.012[a]	0.290±0.023[a]	0.340±0.039[a,b]
TRP-2/β-actin	0.426±0.033	0.591±0.014[a]	0.235±0.010[a]	0.299±0.023[a,c]

Data are presented as the mean ± standard deviation (n=4). [a]P<0.01 vs. the control group; [b]P<005 and [c]P<0.01 vs. the wogonin group. ICI182780, estrogen inhibitor; U0126, extracellular signal-regulated kinase inhibitor; TYR, tyrosinase; TRP-1, tyrosinase related protein 1; TRP-2, tyrosinase related protein 2; ERK1, extracellular signal-regulated kinase 1; ERK2, extracellular signal-regulated kinase 2; JNK2, c-Jun N-terminal kinase 2.

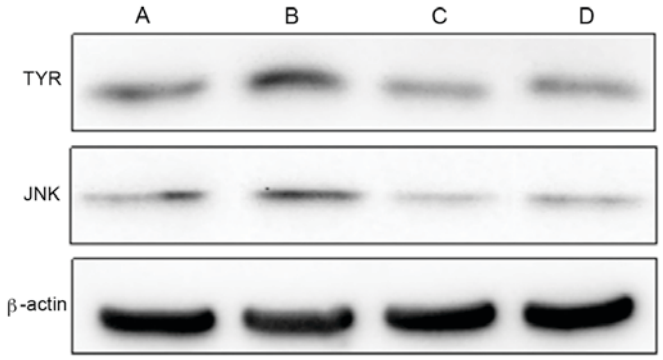

Figure 1. Expression levels of TYR and JNK protein were detected in (A) the control group, (B) the estradiol group, (C) the wogonin group and (D) wogonin + ICI182780 group using western blot analysis. TYR, tyrosinase; JNK, c-Jun N-terminal kinase; control group, cells in the presence of Dulbecco's modified Eagle medium without treatment; estradiol group, cells in the presence of estradiol (10^{-3} μmol/l); wogonin group, cells in the presence of wogonin (10 μmol/l); wogonin + ICI182780 group, cells in the presence of ICI182780 (1 μmol/l) and 10 μmol/l of wogonin.

(P<0.01; Table VI and Fig. 2). ICI182780 was able to reverse the decline of the mRNA expression levels of TYR, TRP-1, TRP-2, ERK1, ERK2 and JNK2 instigated by wogonin, as indicated by the significant increases in mRNA expression levels of TYR, TRP-1, TRP-2, ERK1, ERK2 and JNK2 in the wogonin + ICI182780 group when compared with the TYR, TRP-1, TRP-2, ERK1, ERK2 and JNK2 mRNA expression levels of the wogonin group (P<0.05; Table VI).

Effects of wogonin and U0126 on mRNA expression levels of TYR, TRP-1 and TRP-2 in A375 cells. Results in the wogonin group indicated that wogonin significantly inhibited the mRNA expression levels of TYR, TRP-1 and TRP-2 when compared with the control group (P<0.01; Table VII and Fig. 3). The presence of U0126 resulted in the reversal of declined mRNA expression levels of TYR, TRP-1 and TRP-2 elicited by wogonin, which was suggested by the significant increases in mRNA expression levels of TYR (0.511±0.030), TRP-1 (0.340±0.039) and TRP-2 (0.299±0.023) exhibited in the wogonin + U0126 group when compared with the mRNA expression levels of TYR (0.405±0.018), TRP-1 (0.290±0.023) and TRP-2 (0.235±0.010) in the wogonin group (P<0.05; Table VII).

Discussion

In humans, melanin is the primary determinant of skin color (11), with a greater production of melanin resulting in darker skin pigmentation (12). Melanogenesis is a complicated

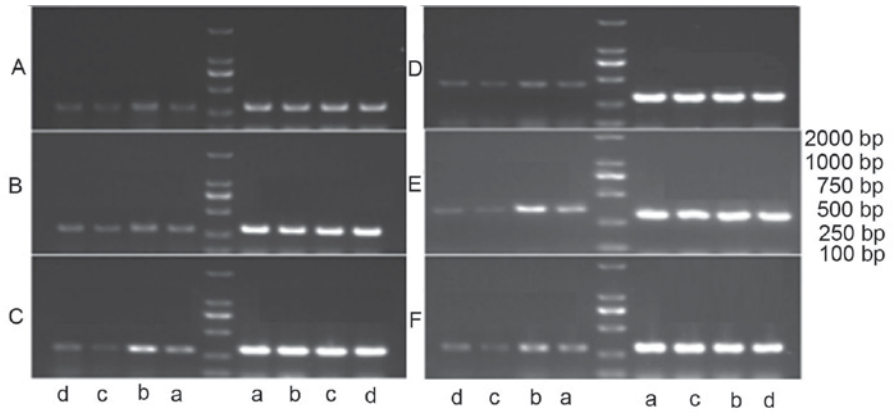

Figure 2. mRNA expression levels of (A) TYR, (B) TRP-1, (C) TRP-2, (D) ERK1, (E) ERK2 and (F) JNK2 were detected in A375 cells by reverse transcription-quantitative polymerase chain reaction. Groups were divided into (a) the control group, (b) the estradiol group, (c) the wogonin group and (d) the wogonin + ICI182780 group. β-actin bands are shown on the right of the ladder. ICI182780, estrogen inhibitor; TYR, tyrosinase; TRP-1, tyrosinase related protein 1; TRP-2, tyrosinase related protein 2; ERK1, extracellular signal-regulated kinase 1; ERK2, extracellular signal-regulated kinase 2; JNK2, c-Jun N-terminal kinase 2; control group, cells in the presence of Dulbecco's modified Eagle medium without treatment; estradiol group, cells in the presence of estradiol (10^{-3} μmol/l); wogonin group, cells in the presence of wogonin (10 μmol/l); wogonin + ICI182780 group, cells in the presence of ICI182780 (1 μmol/l) and 10 μmol/l of wogonin.

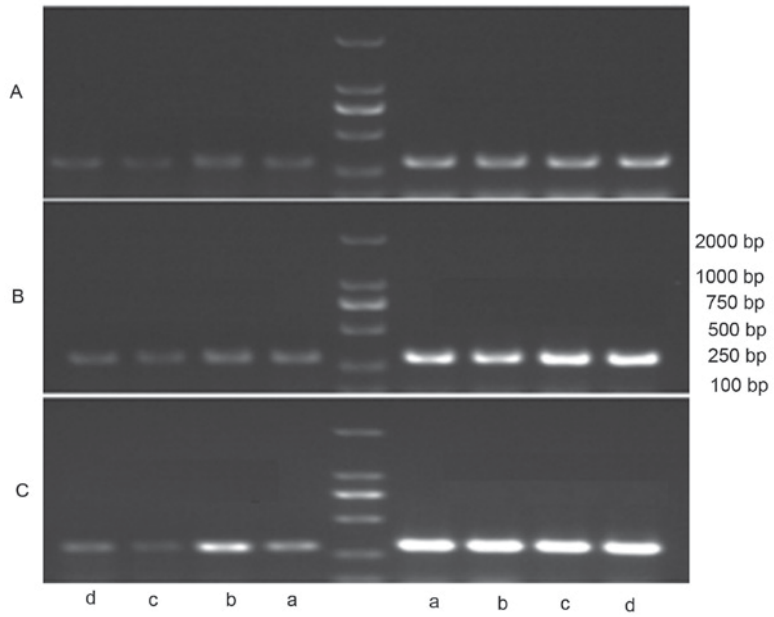

Figure 3. mRNA expression levels of (A) TYR, (B) TRP-1 and (C) TRP-2 in A375 cells were detected by reverse transcription-quantitative polymerase chain reaction in (a) the control group, (b) the estradiol group, (c) the wogonin group and (d) the wogonin + ICI182780 group. β-actin bands are shown on the right of the ladder ICI182780, estrogen inhibitor; TYR, tyrosinase; TRP-1, tyrosinase related protein 1; TRP-2, tyrosinase related protein 2; ERK1, extracellular signal-regulated kinase 1; ERK2, extracellular signal-regulated kinase 2; JNK2, c-Jun N-terminal kinase 2; control group, cells in the presence of Dulbecco's modified Eagle medium without treatment; estradiol group, cells in the presence of estradiol (10^{-3} μmol/l); wogonin group, cells in the presence of wogonin (10 μmol/l); wogonin + ICI182780 group, cells in the presence of ICI182780 (1 μmol/l) and 10 μmol/l of wogonin.

process that occurs within the melanosome of melanocytes and consists of a series of oxidation reactions catalyzed by various enzymes that are regulated by the tyrosinase gene, including TYR, TRP-1 and TRP-2 (13-15). Estrogen is able to bind to the estrogen receptor present in melanocytes and consequently activate the ER-mediated mitogen-activated protein kinase (MAPK) signaling pathway. MAPK is a type of kinase that is specific to serine or threonine and predominantly interacts with three members: JNK, ERK and p38 MAPK (16).

Various studies have demonstrated that the MAPK signaling pathway is involved in the regulation of melanogenesis and has an important role in the regulation of the activity and function of melanocytes (17). Park *et al* (18) indicated that the activated ERK pathway may inhibit melanin synthesis through decreasing the expression of the microphthalmia-associated transcription factor gene, and an alternative study suggested the p38 MAPK pathway may promote melanin synthesis through increasing the expression of the tyrosinase gene (19). Furthermore the JNK pathway may be implicated in the inhibition of melanin synthesis (20). A previous study revealed that phytoestrogen extracted from *Eucommia ulmoides* may inhibit melanocyte proliferation, melanin synthesis and tyrosinase

activity (9); however, the regulation of these mechanisms have not yet been fully clarified.

In the present study, safe doses of wogonin were indicated to significantly inhibit melanin synthesis and tyrosinase activity in A375 cells. Furthermore, this action was reversed by the presence of the estrogen receptor inhibitor, ICI182780, and the ERK inhibitor, U0126. The present findings indicate that wogonin may inhibit melanin synthesis by decreasing tyrosinase activity via the ER-ERK pathway. Western blot analysis revealed that wogonin significantly inhibited the protein expression levels of TYR and JNK in A375 cells, which was reversed by ICI182780. Decreasing the protein expression levels of TYR and JNK may therefore be associated with the regulation of melanin synthesis. RT-PCR showed that wogonin significantly inhibited mRNA expression levels of TYR, TRP-1, TRP-2, ERK1, ERK2 and JNK2, which may be involved in the regulation of melanin synthesis. To conclude, the present study revealed that wogonin may inhibit the synthesis of melanin in A375 cells by inhibiting the expression of TYR, TRP-1, TRP-2 and ERK1, ERK2 and JNK2.

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