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Inhibitory Effect of Active Compounds Isolated from Impatiens balsamina(Garden Balsam) for Melanogenesis

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봉선화 내에 함유 된 레톡시나프타퀴논의 미백활성에 미치는 영향

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본 논문은 피부미백에 관한 실험적 연구로서, 봉선화(Impatiens balsamina Linne)의 성분 중에서 하이드록시-나프타-퀴논(hydroxynaphthoquinone)이 멜라닌 생성억제 효능, 색소침착 저해 효과 등의 미백 활성이 있는 것으로 밝혀졌다. 피부흑화(melanogenesis)는 멜라닌 색소생성세포(melanocyte)에서 자외선 등의 자극에 대한 방어기작으로 멜라닌 생성 활동이 증가되고 이로 말미암아 만들어진 다량의 멜라닌이 각질형성세포(keratinocyte)로 전이되어 피부 표피층에 축척된 결과이다. 비록 멜라닌이 피부에 보호작용을 하나 피부의 과색소 침착은 기미, 주근깨, 피부염증 후의 피부흑화, 노인성 색소반점 등을 일으키며 이로 인해 피부미용 상의 불편뿐만 아니라 정신적으로 부정적인 영향을 미쳐 사회활동에 불편을 초래하기도 한다. 멜라닌 생성 과정은 아미노산의 일종인 티로신(tyrosine)에 티로시나제(tyrosinase)라는 효소가 작용하여 도파(DOPA), 도파퀴논(dopaquinone)으로 바뀐 후 비효소적인 산화 반응을 거쳐 만들어 지며, 이것이 피부 내에 이상 침착하여 기미, 검버섯 등이 생기는 것이라고 알려져 있다. 이와 같은 색소침착, 기미, 반점 등의 완화, 예방 및 치료에는 멜라닌 생성을 억제하는 물질, 예를 들면 하이드로퀴논(hydroquinone), 알부틴, 비타민 C 및 그 유도체 등이 개발되어 사용되고 있으나 이 중, 하이드로퀴논은 일단 효과가 인정되고 있지만 감작성이 있기 때문에 일반적으로 사용이 제한되고 있다. 아스콜빈산은 쉽게 산화되어, 이를 배합한 제품에는 변색, 변취가 되는 문제를 야기하고, 식물추출물 유래의 물질들은 식물의 산지에 따라 효능의 차이가 심하여 제품의 균질성이 유지되기 어렵다. 이러한 이유로 많은 멜라닌 생성을 억제하는 물질을 개발하기 위한 연구들이 계속 진행하고 있다. 2-히드록시-[1,4]나프토퀴논 유도체는 다음과 같은 구조를 나타낸다.

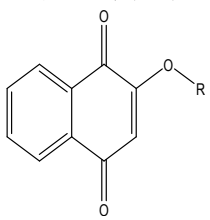

그림 1. 일반 구조식

상기 구조에서 R은 포화 혹은 불포화된 직쇄 또는 분지쇄의 알킬로서, 바람직하게는 C1 내지 C10의 포화 혹은 불포화된 직쇄 또는 분지쇄의 알킬이고, 보다 바람직하게는 C1 내지 C5의 포화 혹은 불포화된 직쇄 또는 분지쇄의 알킬이다. 실험에 활용된 화합물은 다음과 같은 반응식으로 얻어졌다.

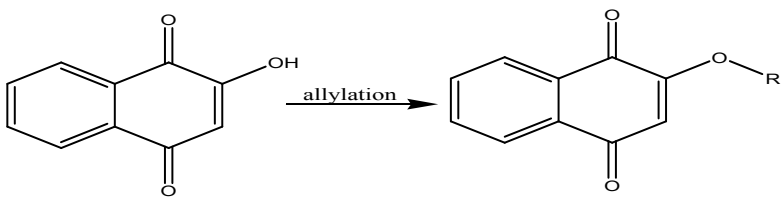

그림 2. 반응식

2-히드록시-[1,4]나프토퀴논과 포타슘카보네이트(K2CO3)를 아세토니트릴에 용해한 후, 저온수조로 냉각하고 알킬할라이드를 적당한 당량비로 천천히 적하하여 히드록시기와 에테르(ether) 반응을 통하여 얻어질 수 있다. 상기 반응식에서 R은 C1~10의 포화 또는 불포화된 알킬기로서 직쇄형 또는 분지쇄형 모두 가능하다. 상기 화합물인 2-히드록시-[1,4]나프토퀴논(2-hydroxynaphthoquinone)은 배양된 쥐의 멜라노마 세포에 대하여 하이드로퀴논과 대등한 멜라닌 생성 억제효과를 보였다. 하이드로퀴논은 저농도에서 강력한 멜라닌 생성 억제효과를 보이지만 상기 화합물들은 보다 낮은 농도에서도 세포독성을 나타내지 않으며 하이드로퀴논보다 높은 멜라닌 생성 억제 효과를 보였다.

Ⅰ. Introduction

The ambient levels of harmful ultraviolet (UV) radiation emitted by the sun are increasing due to destruction of the ozone layer. Therefore, our skin is exposed to more UV radiation and often suffers from various harmful effects of UV. Melaninpigmentation in human skin is a major defense mechanism against UV light of the sun, but abnormal hyperpigmentation such as freckles, chloasma, lentigines and other forms of melanin hyperpigmentation could be serious aesthetic problems.[1)] Therefore, potent active agents for the improvement of hyperpigmentation are sought for their cosmetic use. Many chemicals such as hydroquinone, arbutin, kojic acid, and ascorbic acid are well known for their melanogenic inhibitory functions.[2, 3)] Impatiens balsamina Linne, classified into Balsaminaceae, is annual plant which grows naturally in Southeast Asia including India, Malaysia, China and Korea, and cultivated for ornamental. A flower contained lawsone, lswsone methylether and Although it have some toxicity, contains.[4, 5, 6)] This study was to explore the efficacy of whitening were the following experiment. First, active compounds Methoxynaphtoquinone isolated from Garden Balsam. Next Melanin content assay, Measurement of cell proliferation, Radiometric tyrosinase assay, RNA Preparation and Reverse Transcription -Polymerase Chain Reaction (RT-PCR), Western Blotting Such experiments. And reports the result to get the valence.

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Ⅱ. Materials and Methods

1. Chemicals

All tissue culture medium and components were purchased form GIBCO BRL (Long Island, NY, USA). L-tyrosine, L-Dopa, PMA, MTT, Triton X-100, PMSF, sodium phosphate, -mercaptoethanol, HCl and charcoal were from Sigma Chemical Co. (St. Louis, MO, USA). Protease inhibitor cocktail was from Boehringer-Mannheim (Indianapolis, IN, USA), and L-[U-14C] tyrosine and [3H] tyrosine were from Amersham (Piscataway, NJ, USA).

2. Cells and Culture

Melan-a melanocytes were a kind gift from Dr. D.C. Benette (St. George's Hospital, London). Melan-a cells were originally derived from C57BL mice[7)], and grown in a humidified incubator at 37℃ under 5 % CO2.

Cells were routinely passaged in RPMI 1640 supplemented with 5% fetal calf serum, 100 uM -mercaptoethanol, 50 U/㎖ penicillin, 50 ㎍/㎖ streptomycin, 2 mM L-glutamine and 200 nM PMA.

3. Cell Treatment

Melan-a cells were seeded into 6-well plate at a density of 2.5 x 104 cells per well and allowed to attach for 24 hrs. After then, triplicate cultures were fed with fresh medium containing various concentrations of compound (methoxynaphtoquinone). After 48 hrs, the medium was replaced with the same, fresh test medium. After further 48 hrs, cells are harvested with 0.5 ㎖ of 0.25 % trypsin/EDTA. After dislodgingthe cells with occasional agitation, 2 ㎖ of medium were immediately added to inactivate the trypsin, and 100 ㎕ aliquot were seeded into 96-well plate for MTT assay, as described below. The remainder cell suspensions were centrifuged for 5 min at 1500 g, washed with PBS and then solubilzed in 200 ㎕ of extraction buffer (1% Nonidet P-40, 0.01 % SDS, 0.1 M Tris-HCl pH7.2 and protease inhibitor cocktail). Extracts were solubilized at 4℃ for at least 1 hr and then assays were conducted for each sample, in triplicate.

4. Melanin content assay

After the treatment and extraction as described above, cell extracts were centrifuged for 5 min at 15,000 rpm. The resulting pellets were lysed with 200 ㎕ of 1 N NaOH and transferred to 96-well plate in triplicate. Relative melanin content was determined by absorbance at 405 nm in Power Wave x 340 ELISA reader (Bio-tech Instrumnets, Inc.).

5. Measurement of cell proliferation

MTT assay was used to determine cell proliferation. After the treatment as described above, 100 ㎕ aliquots of harvested cells were plated. Cells were allowed to attach and grow overnight at 3 7℃. The media were discarded, 100 ㎕ of 0.5 ㎎/㎖ MTT was added to each well and incubated at 37℃ for 4hrs. After the incubation, plate was centrifuged for 10 min at 3000 rpm to down the cells. Supernatants were discarded and 200 ㎕ of isopropanol was added and the plate was incubated at RT for 4hrs. The formazan precipitates were quantitated by absorbance at 562 nm in Power Wave x 340 ELISA reader (Bio-tech Instrumnets, Inc.).

6. Radiometric tyrosinase assay

Tyrosinase activity was measured according to the method of Virador with some modifications[8]. Briefly, tyrosinase assay was performed in 96-well plate by adding 30 ㎕ of cell extracts which were prepared as described above, 10 ㎕ of L-[U-14C] tyrosine and 10 ㎕ of 0.25 mM L-Dopa cofactor in 1M sodium phosphate buffer, pH 7.2, containing 0.01% albumin. Reactions were incubated for 4 hrs at 37℃ and stop the reaction by cooling. The contents of each well were transferred to 2.5 cm diameter prelabeled 3MM filter disk and air-dried. The disks were washed three times with 0.1 N HCl containing excess cold L-tyrosine, twice with 95% ethanol and once with acetone. The disks were air-dried and put into liquid scintillation vial, mixed with scintillation cocktail, and the radioactivity was determined by LS 6500 scintillation

system (Beckman, USA).

7. RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Melan-a cells were seeded into T-75 flask at a density of 2 x 105 cells per flask and allowed to attach for 48 hrs. Compounds were treated as described above. Total cellular RNA was prepared using RNAeasy Mini Kit (Qiagen) according to the supplier's instruction. Primers used for RT-PCR analysis in this study were as follows(Table 1).

Table 1. Nucleotide Sequences used in this study

Gene	Primers
tyrosinase	5′TGCCAACGAT CCTATCTT CC3(5′primer) 5′TGAGGAGTGGCTGCTTTT CT3′(3′primer)
tyrosinase-related protein-1 (TRP-1)	5′CCCTTGCGCTTCTTCAAT AG3′(5′primer) 5′TTGCAACATTT CCTGCAT GT3′(3′primer)
tyrosinase-related protein-2 (TRP-2)	5′CCGACTACGT GATCACCA CA3′ (5′primer) 5′TGGCAATTTCATGCTGTTT C3′(3′primer)
glyceroaldehyde -3phosphate dehydrogenase (G3PDH)	5′ATGTTCGTCATGGGTGTGA A′(5′primer) 5′GGGGTCTACAGGCAACTG3′ (3′primer)

These primers were synthesized by Bioneer co., Korea. For cDNA synthesis, 1 g of the total RNA was reverse transcribed in 20 l of reaction mixture containing 2 l of 10x reverse transcription buffer (Perkin-Elmer), 4 l of 25 mM MgCl2 (Perkin-Elmer), 2 l of 10 mM each dNTPs (Clontech), 1l of 50 M Oligo d(T)16 (Perkin-Elmer), 20 units of RNasin (Promega) and 50 units of MuLV reverse transcriptase (Perkin-Elmer). Reverse transcription reaction mixture was incubated at RT for 10 min, 42℃ for 15 min, 99℃ for 5 min, and 5℃ for 5 min (GeneAmp PCR system 2400 thermal cycler, Perkin-Elmer). For PCR amplification of cDNA, 1l of the cDNA product was amplified in a total reaction volume of 50 l containing 5 units of DNA Polymerase(AmpliTaq DNA Polymerase, Perkin-Elmer), GeneAmp 10 x PCR buffer (Perkin-Elmer), 4 l of 25 mM MgCl2 (Perkin-Elmer), 1l of 10 mM each dNTPs (Clontech), 20 pmole upstream primer, and 20 pmole downstream primer. DNA amplification was performed using a Perkin Elmer Gene Amp PCR system 2400 thermal cycler. The PCR cycle conditions were melting for 15 seconds at 95℃, annealing for 30 seconds at 60℃, extension for 90 seconds at 72℃. PCR products were resolved on 1 % agarose gel and visualized by ethidium bromide staining and photographed.

8. Western Blotting

Cells were treated as described above. At the end of each treatment period, cells were washed in PBS and were lyzed in extraction buffer containing 1% Nonidet P-40, 0.01% SDS, and the protease inhibitor cocktail. Protein contents were determined with a BCA assay kit (Pierce, Rockford, IL) and equal amounts of each protein extract (10 ㎍ per lane) were resolved using 8 % SDS polyacrylamide gel (Koma Biotech, Korea), and transblotted onto nitrocellulose membranes (Amersham, Piscataway, NJ) and the membranes were blocked with 5% nonfat milk in TBS buffer. Following the blocking, the membranes were incubated with PEP7(anti-Tyrosinase), PEP1(anti-TRP1), or PEP8(anti-TRP2) (each at a 1:1000 dilution).

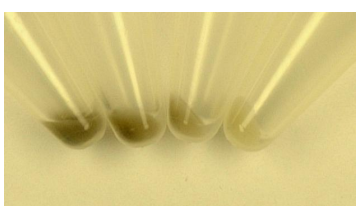
PEP7, PEP1, and PEP8 were kind gifts from Dr. Vincent J. Hearing (NCI, NIH). The membranes were then incubated with HRP-conjugated anti-rabbit IgG at a dilution of 1:2000. Immunoreactive bands were detected with enhanced chemiluminescence using an ECLkit (Amersham, Piscataway, NJ) according to the manufacturer's instructions.

Ⅲ. Results

1. Effect of methoxynaphtoquinone on Pigmentation of melan-a cells

Methoxynaphtoquinone showed the remarkable whitening effect on melan-a cells (Fig 1 A). This visual determination showed highly related results with direct measurement of melanin content (Fig B). At a concentration of 0.1 ㎍/㎖ and 0.2 g/㎖ of methoxynaphtoquinone, melanin content decreased by almost 50% and 70%, respectively. These are very significant decrease in melanin contents when compared with other melanogenic inhibitors (data not shown).

A



0 0.05 0.1 0.2

B

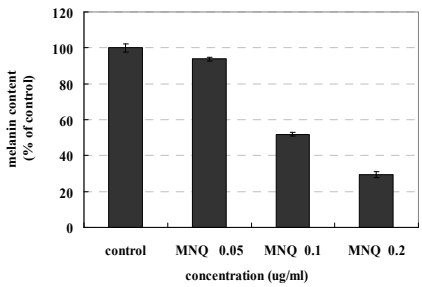


Figure 1. Methoxynaphtoquinonedecreased the pigmentation of Melan-a cells. Melan-a cells were treated with or without methoxynaphtoquinone. After 4days, cells were harvested. (A) Harvested cells were pelleted and photographed. (B) Their melanin contents were assayed as described in Materials and Methods. Melanin contents were expressed as percent of control. Values are the averages of three determinations±SD.

2. Effect of methoxynaphtoquinone on cell proliferation

When selecting whitening compounds, one of important points is that they should have minimal effect on melanocyte cell proliferation. Thus, the proliferation of cells treated with methoxynaphtoquinone was evaluated by MTT assay. Methoxynaphtoquinone showed little inhibitory effect on cell proliferation at the tested concentration (Fig 2).

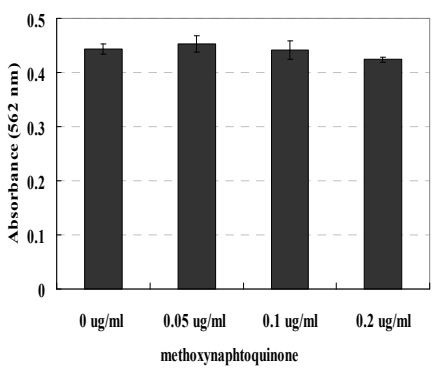


Figure 2. Methoxynaphtoquinone did not show any inhibitory effect on cell proliferation. Melan-a cells were treated with or without methoxynaphtoquinone. After 4 days, cells were harvested. MTT assay was performed as described in Materials and Methods. Values are the averages of three determinations±SD.

3. Effect of methoxynaphtoquinone on tyrosinase activity

Tyrosinase is the rate-limiting enzyme in melanin synthesis, and some melanin production-inhibiting agents such as arbutin and kojic acid are known to inhibit the tyrosinase activity.[9] To determine the effect of methoxynaphtoquinone on tyrosinase activity, we measured tyrosinase activity by counting [14C] tyrosineincorporation into nascent insoluble melanin as described in Materials and Methods. Compared with the untreated control, methoxynaphtoquinone reduced tyrosinase activity by 22% and 33% at 0.1g/㎖ and 0.2 ㎍/㎖, respectively (Fig 3). To examine whether this reduced tyrosinase activity is caused by direct inhibition of tyrosinase action, we performed tyrosinase assay using solubilized tyrosinase as described in Materials and Methods. Because methoxynaphtoquinone showed no inhibition on solubilized tyrosinase, we concluded that methoxynaphtoquinone is not direct inhibitor of tyrosinase(data not shown).

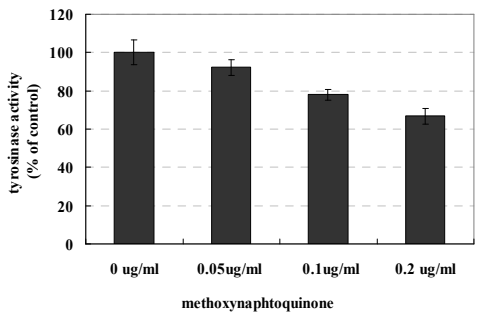


Figure 3. Methoxynaphtoquinone inhibited tyrosinase activity. Melan-a cells were treated with or without methoxynaphtoquinone . After 4 days, cells were harvested. Radiometric tyrosinase assay was performed as described in Materials and Methods. Changes in tyrosinase activity were expressed as % of control. Values are the averages of three determinations±SD.

4. Effect of methoxynaphtoquinone on expression of tyrosinase, TRP-1 and TRP-2

To explore the mechanism responsible for the decreased pigmentation, we examined changes in the mRNA levels and protein levels of three important melanogenic enzymes (tyrosinase, TRP-1 and TRP-2) using RT-PCR and western blotting, respectively. Melan-a cells were treated with 0.1 g/㎖ and 0.2 g/㎖ of methoxynaphtoquinone for 4 days and then, each mRNA level and protein level was examined. Fig. 4 shows methoxynaphtoquinone did not inhibit tyrosinase, TRP-1 and TRP-2 mRNA and protein expression. These results suggest that methoxynaphtoquinone might reduce tyrosinase activity not by regulation of the three important melanogenic enzymes but by any other mechanisms. Further mechanism studies are in progress.

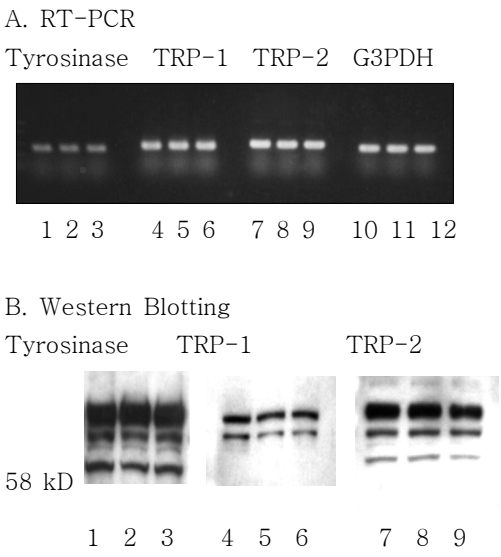


Figure 4. Methoxynaphtoquinone had no effect on tyrosinase, TRP-1 and TRP-2 expression. Fig A. Total RNA was extracted and applied to RT-PCR as described in Materials and Methods .Gel electrophoresis of the RT-PCR products. Lane 1, 4, 7, 10 ; PCR product of tyrosinase (400bp), TRP-1 (450bp), TRP-2 (500bp) and G3PDH (400bp) of untreated control, respectively : Lane 2, 5, 8, 11 ; PCR product of tyrosinase, TRP-1, TRP-2 and G3PDH of 0.1 ug/ml methoxynaphtoquinone treated melan-a, respectively ; Lane 3, 6, 9, 12: PCR product of tyrosinase, TRP-1, TRP-2 and G3PDH of 0.2 ㎍/㎖ methoxynaphtoquinonetreated melan-a, respectively. (B) Total protein was extracted and applied to western blotting as described in Materials and Methods. Specific detection of tyrosinase, TRP-1, TRP-2 was performed using PEP7(anti-Tyrosinase), PEP1(anti-TRP1), or PEP8(anti-TRP2). Lane 1, 4, 7 ; tyrosinase , TRP-1 and TRP-2 of untreated control, respectively ; Lane 2, 5, 8 ; tyrosinase, TRP-1and TRP-2 of 0.1 ㎍/㎖ methoxynaphtoquinone treated melan-a, respectively ; Lane 3, 6, 9 ; tyrosinase, TRP-1 and TRP-2 of 0.2 ㎍/㎖ methoxynaphtoquinonetreated melan-a, respectively.

Ⅳ. Discussion

Balsam's constituents, such as this Anthocyanin,[10] cyanidin, delphinidin, pelargonidin, malvidin,[11] lawsone[12] isolated from flower and β-amyrin, α-spina-sterol, balsamina sterol, sitosterol, tricholoside planteose and hosenkol-A from seed.[13, 14, 15, 16] Also John et al[17] reported the anti-fungal activity of 2-methoxy-1,4-naphthoquinone against Rhodotrula glutinis. and Watery extract (1:3) inhibit the growth of Trichophyton, Schonlein's purpurea in vitro and its decoctions partially inhibit the growth of Staphylococcus aureus, Streptococcus hemolyticus, Pseudomonas aeruginosa and Typhus enteritis.[18] In folk remedy, athletes region have been treated by washing with boiled water of total plant. Galenic pharmacy in oriental medicine delineates that the cormophyte called Ji-Gab-Cho is effective in inflammation, blood flow and edema improvement and remittance of dyscinesia mediated by pain of musculoskeletal system, and treats contusion, tuberculous lymphadenitis, infectious skin diseases, phlegmone, hematochezia and metrorrhagia.[19, 20] Beside mentioned above, the seed called Geub-Seong-Ja is used to uterine contraction and hysteromyoma, and confirmed contraception activity in Rat.[21, 22] Hydroquinone[23] (HQ) is one of the most effective inhibitors of melanogenesis in vitro and in vivo, and is widely used for the treatment of melanosis and other

hyperpigmentary disorders. In an attempt to get some insight into the molecular mechanism of the depigmenting action, which is still very poorly understood, we have investigated the effect of HQ on the tyrosinase catalysed conversion of tyrosine to melanin. Incubation of 0.5 mM tyrosine with 0.07 U/㎖ tyrosinase in phosphate buffer at pH 6.8 in the presence of 0.5 mM HQ led to no detectable melanin formation, due to the preferential oxidation of HQ with respect to tyrosine (HPLC evidence). Kinetic investigations showed that HQ is a poorer substrate of tyrosinase than tyrosine ; yet, it may be effectively oxidised in the presence of tyrosine owing to the generation of catalytic amounts of dopa acting as cofactor of tyrosinase. Product analysis of HQ oxidation with tyrosinase in the presence of dopa showed the predominant formation in the early stages of hydroxybenzoquinone (HBQ), arising from enzymic hydroxylation and subsequent oxidation of HQ, along with lower amounts of benzoquinone (BQ). These results suggest that the depigmenting activity of HQ may partly be related to the ability of the compound to act as an alternate substrate of tyrosinase, thereby competing for tyrosine oxidation in active melanocytes. As a result of the experiment is as follows. At a concentration of 0.1 ㎍/㎖ and 0.2 g/㎖ of methoxynaphtoquinone, melanin content decreased by almost 50% and 70%, respectively(Fig B). Methoxynaphtoquinone showed little inhibitory effect on cell proliferation at the tested concentration (Fig 2). Methoxynaphtoquinone reduced tyrosinase activity by 22% and 33% at 0.1g/㎖ and 0.2 ㎍/㎖, respectively (Fig 3). but Methoxynaphtoquinone did not inhibit tyrosinase, TRP-1 and TRP-2 mRNA and protein expression(Fig 4). These results suggest that methoxynaphtoquinone might reduce tyrosinase activity not by regulation of the three important melanogenic enzymes but by any other mechanisms.

Ⅴ. Conclusion

A conclusion based on experimental results as follows

1. Methoxynaphtoquinone showed the remarkable whitening effect on melan-a cells and concentration of 0.1 ㎍/㎖ and 0.2 g/㎖ of methoxynaphtoquinone, melanin content decreased by almost 50% and 70%.
2. The proliferation of cells treated with methoxynaphtoquinone was evaluated by MTT assay. Methoxynaphtoquinone showed little inhibitory effect on cell proliferation at the tested concentration.
3. Compared with the untreated control, methoxynaphtoquinone reduced tyrosinase activity by 22% and 33% at 0.1g/㎖ and 0.2 ㎍/㎖.
4. Methoxynaphtoquinone did not inhibit tyrosinase, TRP-1 and TRP-2 mRNA and protein expression.

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