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Nelumbo nucifera Leaf Extracts Inhibit Melanogenesis in B16 Melanoma Cells and Guinea Pigs through Downregulation of CREB/MITF Activation

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Abstract *Nelumbo nucifera* leaves extracts (NLE) has been suggested to provide antioxidant, anti-obesity and anticancer effects. However, the research on the anti-melanogenetic effect of NLE was little. Here, we reported that NLE and Gallic acid (GA, major ingredients of NLE) exhibit the hypopigmentary effect in B16F1 cells. NLE and GA showed potent inhibitory effects on tyrosinase, microphthalmia-associated transcription factor (MITF), and tyrosinase-related protein-1 (TRP-1) protein production. The phosphorylation of intracellular protein kinase A (PKA) and cAMP response element-binding protein (CREB) were also decreased, revealing potent anti-melanogenic effects of NLE and GA. However, NLE showed the better effect than GA on reducing melanin formation, implying the importance of the synergism of polyphenolic compounds of NLE. Furthermore, NLE inhibited skin melanogenesis and epidermal hyperplasia of guinea pigs caused by irradiation through attenuating ERK and CREB activation. NLE reduced skin melanogenesis and epidermal hyperplasia induced by UVB in guinea pigs through downregulation ERK and CREB pathways, and the following decreasing of MITF, tyrosinase and TRP-1 expression. These results demonstrated the potential which NLE as an ingredient of hypopigmentary cosmetics.

Keywords: *CREB, gallic acid, melanogenesis, nelumbo nucifera leaves extracts (NLE), tyrosinase*

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1. Introduction

Cutaneous melanin synthesized from melanocyte is essential for determination of skin color and protecting human skin against UV radiation [1]. Melanocytes are situated at the stratum basal layer of the epidermis. After irradiation exposure, melanosomes produce and release melanin to the keratinocytes, and migrate to the outer layer of the skin by degrees. Tyrosinase is the rate-limiting reaction enzyme in melanogenesis [2]. Firstly, UV light exposure induces the keratinocytes to produce α-melanocyte stimulating hormone (α-MSH), which indirectly increases the production of melanin. Following, cAMP expression is increased when α-MSH binds to melanocortin 1 receptors (Mc1r). cAMP triggers the phosphorylation of the CREB transcription factor, thereby promoting MITF activation. MITF binds to the promoter regions of TRP-1 and tyrosinase genes, and regulate their transcription. Besides, cAMP also regulates the ERK pathway to influences the synthesis of melanin.

Severe dermatological problems arise when the over production and accumulation of melanin, such as melanosis, freckles, ephelides, senile lentigines, inflammation, and cancer due to prolonged exposure to UV light [3,4]. How to control the melanngenesis is an important strategy for therapy of pigmentation derived problems. The most common effect of skin whitening agents on the marker is as a tyrosinase inhibitors. Arbutin and kojic acid are the well-known natural tyrosinase inhibitors used as cosmetic ingredients. However, it is somewhat controversial to be used in the skin-whitening products, because the carcinogenic

latent and weak whitening effect of kojic acid are always criticized [5].

Many traditional herbs have been utilized as natural substances for developing whitening cosmetics because of their abilities of preventing melanin biosynthesis by directly inhibiting tyrosinase activity and relatively few side effects [6,7]. Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is a natural polyphenol antioxidant existed in a large number of plant extracts. Previous studies have demonstrated that GA is provided with various biological properties, such as anti-inflammation, antiradical, anticancer, and depigmentation [8,9]. Current studies investigated the inhibitory effect on melanogenesis of many plains rich in GA, such as Dendrobium tosaense, rape bee pollen and Stewartia pseudocamellia leaves [10,11,12].

In decade, our laboratory dedicated to the research on the health care of *Nelumbo nucifera* leaves. We found that GA is the major ingredients of *Nelumbo nucifera* leave extracts (NLE) via HPLC analysis, and demonstrated that NLE improved hepatic injuries, atherosclerosis, obesity, breast cancer, diabetes, and hepatocarcinogenesis [13,14,15,16,17,18]. In this study, we attempted to confirm the anti-melanogenesis of NLE and GA in B16F1 melanoma cells, and we revealed that NLE exerted a better inhibition of melanogenesis than GA. In addition, we verified the effects of NLE and GA on melanin formation in B16F1 cells with regard to CREB/PKA pathway activation. The anti-melanogenic activity of NLE was also studied in guinea pigs model.

2. Materials and Methods

2.1. Preparation of NLE

NLE was prepared from dry lotus leaves using our previously research [16]. The lotus (*Nelumbo nucifera* Gaertn) leaves were washed with distilled water, air-dried at 50 °C, and grinded into powder. 5 L distilled water was prepared to resuspend the 200 g powder at 4 °C for 24 h. The precipitate was removed by filtration and supernatant was condensed using vacuum concentrator. The condensed solution was lyophilized as NLE and was frozen at -80 °C until use. The yield of dry NLE powder was about 17.5%.

2.2. Cell Culture

The murine melanoma B16F1 cells (BCRC60032) were purchased from the Food Industry Research and Development (BCRC, Hsin-Chu, Taiwan). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin G, and 100 mg/mL streptomycin. All cells were cultured at 37 °C and 5% CO_2 condition.

2.3. Cell Viability

The cytotoxicity of B16F1 cells treated with NLE or GA was determined by MTT assay. Briefly, various concentrations of NLE (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) or GA (0, 60, 70, 80, 90, and 100 μM) were treated with B16F1 cells ($3x10^4$ cell/mL) and incubated for 24, 48, and 72 h. The cells were then reacted to MTT (Sigma Aldrich, St.Louis, MO, USA) solution (5 mg/mL in PBS) for 3 h. After media were removed, the isopropanol (Junsei, Tokyo, Japan) was added to dissolve the formazan crystals. The color products were measured by spectrophotometer at 563 nm. The experiments were performed in triplicate.

2.4. Animal Experiments

Female guinea pigs (N:HARTLEY) (weighing 300-350 g, n=4) were acquired from the National Laboratory Animal Center (Taipei, Taiwan). The guinea pigs were acclimated to the laboratory environment for 7 days in a temperature- and humidity-controlled room (22 ± 2°C, 50 -55% humidity) with 12 h light/dark cycles. Animal experiment was approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMU). The animal license of the laboratory (no. 1606) was issued by the CSMU. During the period of experiment, the mice were allowed to take food and water at labium. The animals weighing approximately 500 g were exposed to the UVB radiation (Spectroline Select™ Series, Philips TL/12 lamp emitting 280–305 nm) three times a week for two weeks. The total UVB dose was 500 mJ/cm^2 per exposure. The guinea pigs were anesthetized with isoflurane, and divided regions (length; 1.5 cm, width; 1.5 cm) of the back of each guinea pig were accepted the UVB exposure. 1 or 2% NLE mixed with PEG-40 (First Chemical Manufacture Co., Ltd, Taipei, Taiwan) were given topically to the UV-irradiated regions at next day. Skin biopsies were collected and the skin homogenate was assayed by using hematoxylin and eosin stain, Fontana-Masson stain methods, and measurement of melanin contents.

2.5. Melanin Content Assay

1×10^5 B16-F1 cells were seeded in 6-well plate and treated with 10 μM α-MSH and various concentrations NLE or GA for 24, 48, and 72 h. 200 μL of the medium was measured the absorbance at 405 nm using spectrophotometrically. The cells were washed with ice-cold PBS twice, then lysed with RIPA buffer containing Tris-buffered saline, 0.5% deoxycholate, 0.1% SDS, 1% Triton X-100 and protease inhibitor mixture (Roche Applied Science), then centrifuged at 12,000 × g for 10 min. 200 μL of 1 M NaOH was used to solubilize the pellets at 70°C for 2 h, the absorbance was measured at 405 nm using spectrophotometrically. The supernatants were used to determine protein concentration. Melanin content was measured by normalizing the total melanin values with protein content (μg of melanin/mg of protein).

2.6. Immunoblotting Assay

The cells were solubilized with RIPA buffer and Bradford dye reagent (Bio-Rad) was used to measure the protein concentration. Aliquots of cell lysates (50 μg of protein) were separated by 8-12 % SDS-polyacrylamide gel electrophoresis, then transferred to NC membranes (nitrocellulose membranes). When blocking with non-fat milk for 1 h, the membranes were treated with several primary antibodies overnight at 4°C. Membranes were

washed and subsequently incubated with the secondary antibodies at room temperature for 1 h. The membranes were added to the chemiluminescence solution (ECL; Amersham Biosciences) and detected the antibody complexes in a LAS-4000 Luminescent Image Analyzer (Fujifilm Corporation, Tokyo, Japan) for 1 min. The intensity of each antibody complex was examined by using Fujifilm Multi Gauge V2.2 software.

2.7. Histological Skin Analysis

According to our previously procedures, skin biopsy specimens were dehydrated and embedded in paraffin after fixed in 10% neutral formalin solution at 4°C for 24 h. For histopathological examination, 3 μm thickness skin tissue from different groups were stained with hematoxylin and eosin. Ten different fields per slide of stained skin sections were examined using the light microscope (Nikon, Tokyo, Japan).

2.8. Fontana-Masson Stain

Fontana-Masson staining kit (IHC WORLD, GA, USA) was used to determine the melanin content. The dorsal skin of guinea pigs were firstly fixed in 10% neutral formalin solution for 24 h and embedded in paraffin. 4 μm sections were stained with Fontana-Masson staining solution. Ten different fields per slide were pictured under a light microscope (Nikon, Tokyo, Japan).

2.9. Statistical Analysis

The quantitative data were showed as mean ± SD fort three to ten times of independent examination for each sample. Statistical contrasts between different groups were assayed by one-way analysis of variance (ANOVA) with Tukey' multiple comparisons test. Comparisons between the two groups of samples were assayed by unpaired Student's t-test. The statistical significance was stipulated for *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

3. Results

3.1. NLE and GA Decreased Melanin Biosynthesis in B16F1 Melanocytes

B16F1 cells derived from C57BL/6 mice can produce melanin induced by α-MSH. Therefore, B16 cells are widely used in melanogenesis and whitening studies [19,20]. Firstly, whether the NLE and GA reduced the B16F1 cells survival was detected by MTT assay. Cells were treated NLE or GA for 24 h, no significant cytotoxicity was observed for the NLE when the concentration is below 0.5 mg/mL (Figure 1A). Similarly, 100 μM GA did not lead the B16F1 cells death. Both natural products displayed little toxicity to the B16F1 cells when treatment for 72 h. NLE showed the inhibition rate of 18.1-21.5% in the 0.1-0.5 mg/mL, and the GA expressed 9.8-21.2% in the 60-100 μM. Next, to investigate the effect of NLE and GA on melanin formation, B16F1 cells were stimulated by α-MSH with or without NLE or GA. Images of B16F1 cell pellets performed a notable reduction in the melanin content from 48 h of treatment with NLE or GA in cells in the presence or absence of α-MSH stimulation (Figure 1B). NLE significantly decreased the melanin formation in α-MSH conditions in melanoma cells after 72 h of treatment (Figure 1C). As similar, NLE and GA also abolished the increase of melanin secretion stimulated by α-MSH for 72 h treatment in culture medium. In the basal condition, NLE showed less inhibitory ability regarding the melanin content than GA. However, NLE presented the better efficacy on inhibiting melanogenesis stimulated by α-MSH than GA.

3.2. NLE and GA inhibited Melanogenesis Related Enzyme Levels in B16F1 Cells

The effects of NLE and GA on regulating melanin synthesis induced by α-MSH B16F1 cells were shown in Figure 2. The expression of tyrosinase, MITF and TRP-1 proteins was meaningfully increased by α-MSH stimulation. Following treatment with NLE, the levels of tyrosinase, MITF and TRP-1 proteins decreased in a dose-dependent manner (Figure 2A), indicating that NLE reduced the melanin content via the downregulation of MITF and TYR family proteins. Melanin synthesis has been described to be regulated by the CREB and PKA phosphorylation. Next, the phosphorylation activity of CREB and PKA proteins were examined. In Figure 2A the total amount of the CREB protein was no change after treatment with α-MSH or NLE. α-MSH enhanced CREB and PKA phosphorylation, whereas NLE significant inhibited the CREB and PKA phosphorylation in both basal and stimulated conditions. Unsurprising, the protein contents of MITF, TRP-1, phosphorylated CREB and PKA were inhibited after GA treatment (Figure 2B). However, low dose GA (80μM) induced CREB and PKA proteins phosphorylation in basal condition. These results indicated that NLE had more efficacy than GA on inhibiting melanin synthesis via the CREB/PKA pathway in stimulated condition.

3.3. NLE Ameliorated the Hyperpigmentation Induced by UVB in Guinea Pigs

Following, 1 and 2 % NLE were used in animal model to assess whether NLE has a depigmentation effect. According to the results of H&E staining, compared with control area, NLE reversed the UVB-induced epidermal hyperplasia (Figure 3A), indicating that NLE may ameliorate the UVB-induced skin thickening and damage. Next, melanin contents were detected through Fontana-Masson staining method which stains melanin granules (melanosomes). As the results performed in Figure 3B, UVB induced melanogenesis (black arrows), and NLE decreased melanin content in the epidermis of the guinea pigs. To further confirm this, melanin content of different treated areas was assayed. Figure 3C results showed that NLE reversed the UVB induced the melanin formation to the control level. Finally, we analyzed the effect of NLE on melanogenic proteins expression in skin tissue from guinea pigs. The western blot analysis (Figure 4A) demonstrated that UVB increased the expression of melanogenesis related proteins such as

MITF, tyrosinase, and TRP-1. NLE downregulated the levels of these proteins in UVB-stimulated condition. Furthermore, the c-AMP mediated PKA signaling was impacted by NLE (Figure 4B). In addition, NLE was able to modulate the ERK activity which is involved in regulating melanogenesis. Comprehensive, these results demonstrated that NLE had a depigmenting efficacy in guinea pigs.

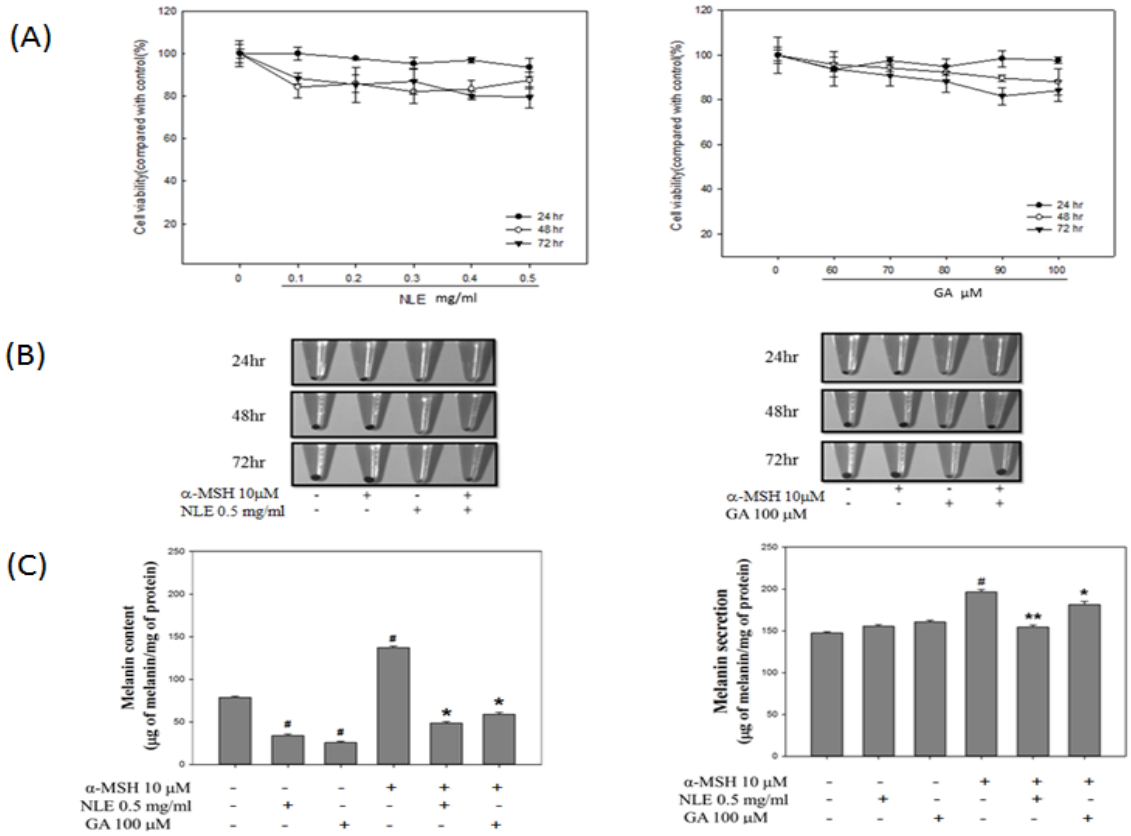

Figure 1. NLE and GA decreased melanin content and secretion B16F1 cells induced by α-MSH

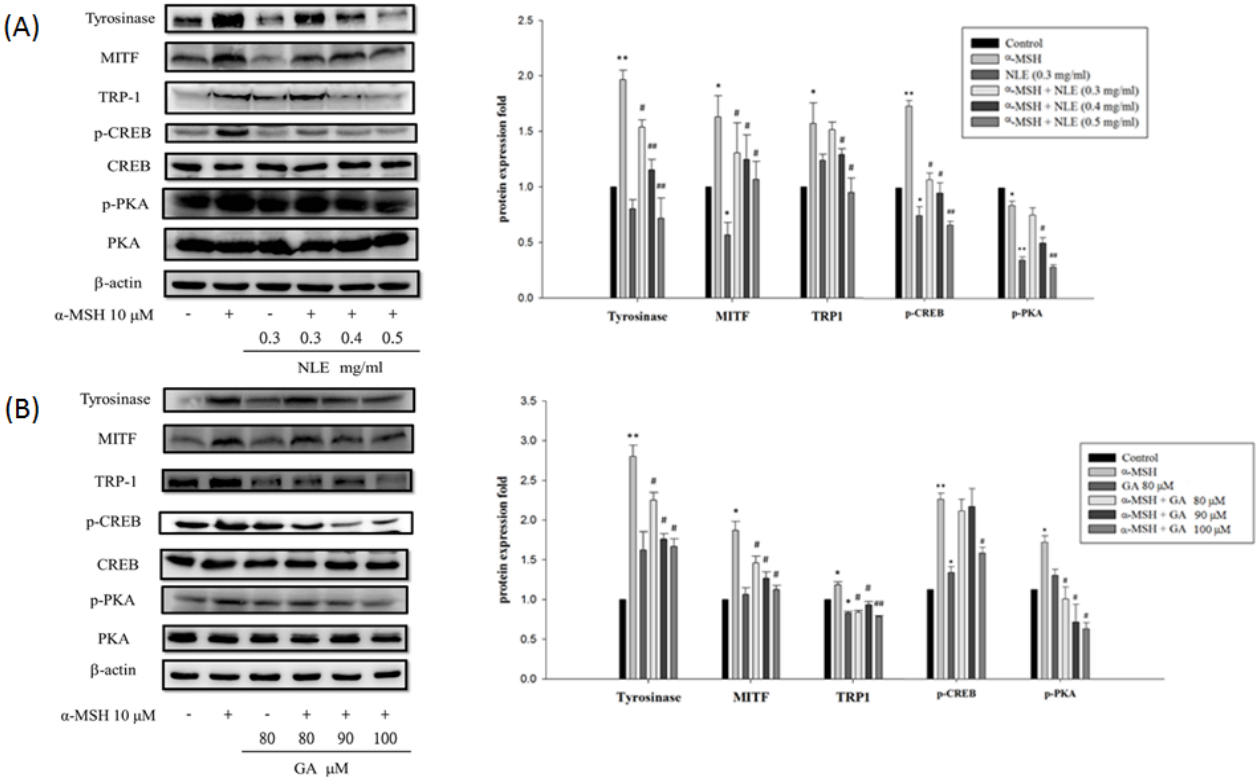

Figure 2. NLE and GA downregulated the melanogenic enzyme expression of B16F1 cells induced by α-MSH

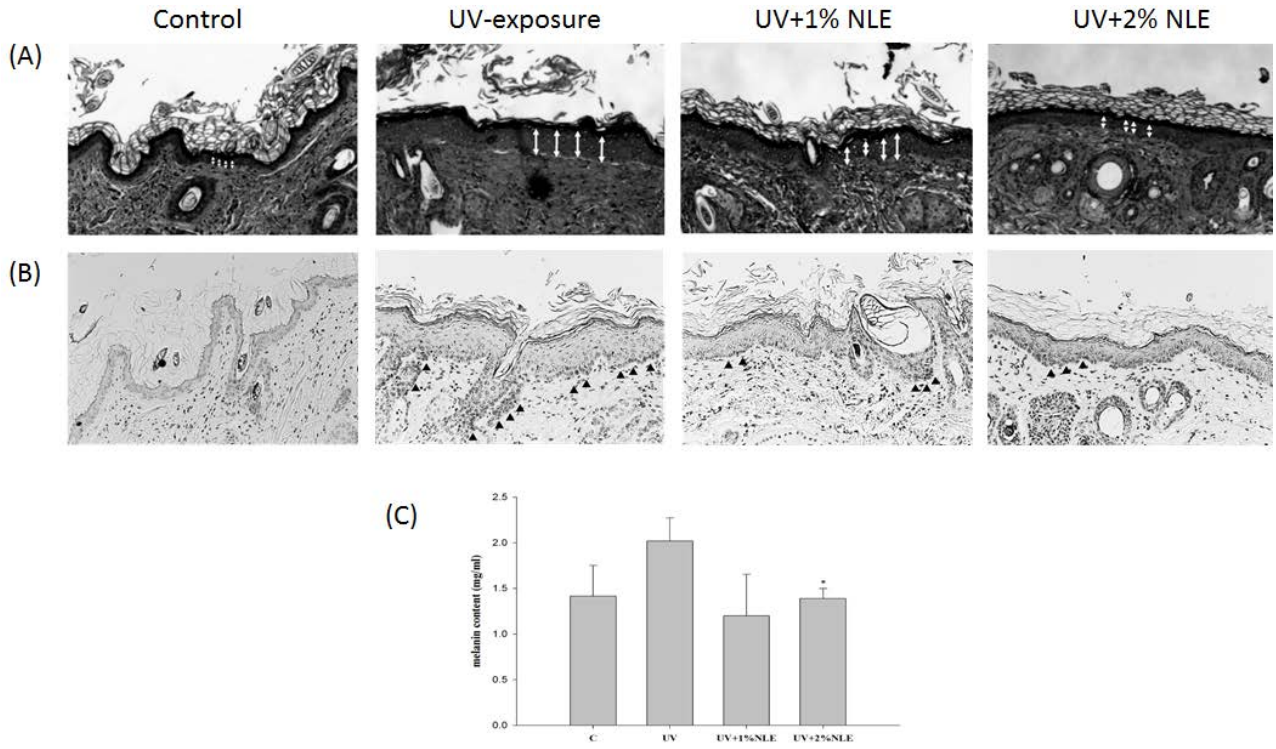

Figure 3. NLE attenuated melanin accumulation of UVB-irradiated skin tissues from guinea pigs

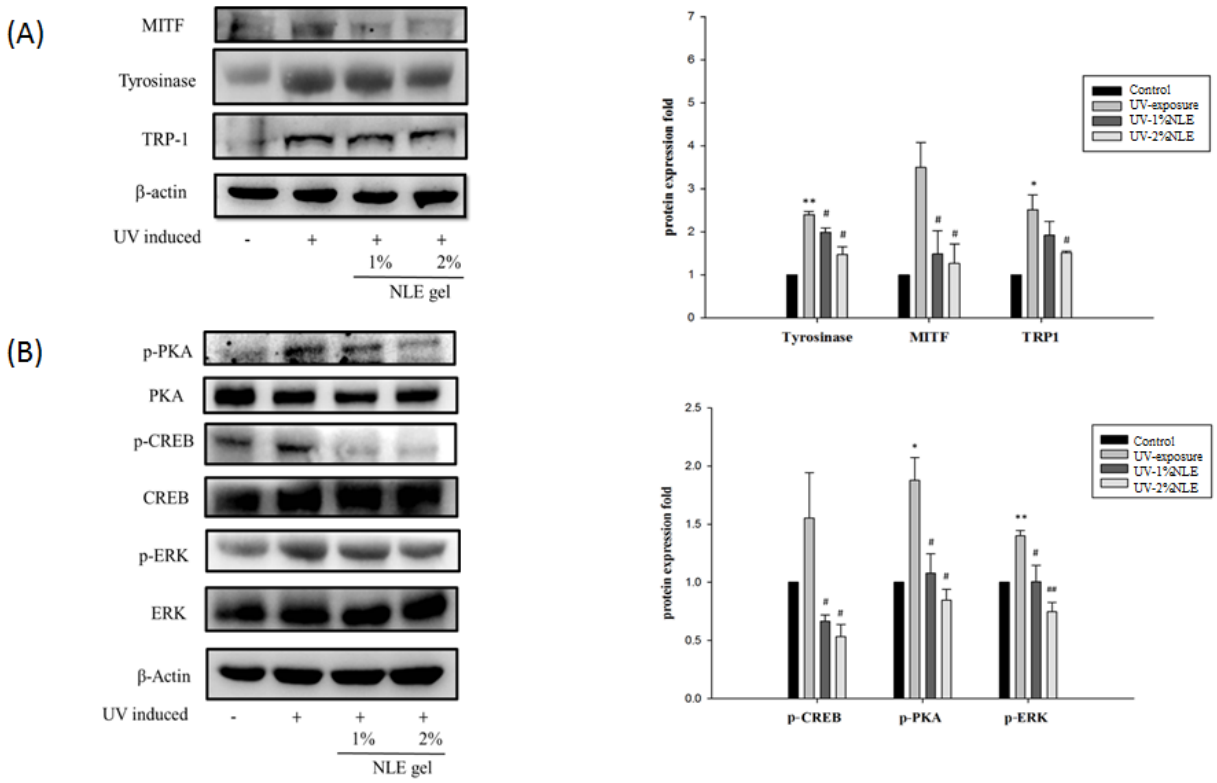

Figure 4. Mechanism of NLE on regulating melanogenic proteins expression of UVB-irradiated skin tissues from guinea pigs

4. Discussion

Nelumbo nucifera is a kind of Asian aquatic plant. Whole parts of *Nelumbo nucifera* are well been used in Asian cooking or for various medicinal purposes. Traditionally, the leaves extracts has been endued with anti-oxidant, anti-inflammation and anti-cancer properties [21,22,23]. Here, this is the first report concerning the effect of *Nelumbo nucifera* leaves potency on melanogenesis. Tyrosinase plays a crucial role in the melanin biosynthesis pathway. In our study, NLE was found to inhibit tyrosinase through MITF pathway in vitro and in vivo.

The activation of the transcription factor CREB is a critical role in the regulation of melanogenesis in human and mice [24]. The depigmenting effects of GA through

down-regulating the CREB signaling are also well demonstrated in vivo and in vitro researches [25,26]. GA is one kind of cosmetic ingredients for skin lightening and pigmentation reduction [27]. The combination of GA and the linoleic acid is often to be used in clarifying spot and speckle of skin. A cosmetic product containing gold nanoparticles combined with GA and protocatechuic acid to prevent skin aging were also reported. Base to the potential of GA on inhibiting the skin damage caused by UVB, a formula containing GA and other polyphenolic compounds could be suitable and useful in the improvement of skin disorder.

Noteworthy, our study demonstrated the better efficacy of NLE on melanogenesis reduction than GA (Figure 1). HPLC analysis was used to analyze the components of NLE in our previous study. The amount of polyphenol compounds is nearly to 0.034 mg/mL NLE extract and six phenolic compounds were identified [16]. Recently researches indicated that two major compounds of NLE involved in the melanogeiesis were GA (6.11 ± 0.15 μg/mg NLE) and catechin (7.42 ± 0.69 μg/mg NLE). After calculation, 0.5 mg/mL NLE used in this study contained 17.95 μM GA and 12.8μM catechin. The inhibitory effects of NLE on melanin formation was better than 100 μM GA treatment alone, indicating the synergic benefit of natural plant extracts. Indeed, catechin, another major compound of NLE, was also reported that 100 μM of catechin inhibited UVB and oxidative stress and JNK activation to enhance human keratinocyte survival [28]. The Bamboo Stems extracts containing catechin showed a significant inhibitory effect on PKA/CREB pathway, then suppress melanin production [29]. Rhodiola sachalinesis which active compounds including catechin suppress melanin production, whereas did not affect CREB phosphorylation or tyrosinase expression, suggesting different effects on melanogenesis [30]. The mechanisms involved in the synergism of natural polyphenolic compounds of NLE deserve extensive investigation.

Recent researches reveal that using a whole plant extracts containing a group of phytochemical working together is more beneficial than using a single compound to achieve a specific effect [31]. The structurally diverse phytonutrients from plant may possess complementary and overlapping mechanisms of potential disease-preventive action. Further, combination of similar foods containing same phytochemicals serves more capacity on health care compared with individual food. Synergistic interactions are of vital importance in phytochemicals, but it presences several difficulties, such as how the phytochemicals in these studies interact with each other and how the interactions lead to synergistic effects. This has led to be questioned to dismiss these medicines as placebos in the absence of clinical proof. On the other hand, whether in vitro and in vivo results of phytochemicals on synergistic interaction have the consistence should be explored. For instance, it is well known that MITF is regulated by CREB-dependent signaling pathway, Wnt/GSK pathway and ERK/MAPK signaling [1]. In this study, NLE decreased melanin formation by attenuating both CREB and ERK phosphorylation in vivo. However, in vitro results showed that NLE inhibited melanogenesis via CREB pathway but no ERK pathway involved in (data not shown). Indeed, in vitro study models are simplistic and useful in verifying outcomes of the finding in vivo. However, it is limited on the complexity of in vitro models and inability to answer specific situation related to human digestion biochemistry [32].

5. Conclusion

NLE and GA inhibited the melanogenesis induced by α-MSH in B16F1 melanoma cells via CREB pathway. The lightening effects were observed following treatment of NLE to UV-stimulated dorsal skin of guinea pigs. According to HE staining assay, the effects of NLE on depigmentation was due to depletion of melanin formation. NLE reduced melanin formation by inhibiting MITF, tyrosinase and TRP-1 activities. Our future study is to identify the therapeutic effects of NLE against UVB exposures in human skin.

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Statement of Competing Interests

The authors have no competing interests.

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