Effects of pine needle essential oil on melanin synthesis in B16F10 cells and its mechanism

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Abstract. The present study aimed to investigate the melanin synthesis effect of pine needle essential oil (PNEO) extracted using microwave-assisted extraction on α-MSH-induced B16F10 melanoma cells and its molecular mechanism of action. Cell Counting Kit-8 was used to measure the safe concentration of B16F10 cells after 24 and 48 h post-treatment. Tyrosinase (TYR) activity assay was used to examine intracellular enzyme viability; western blotting (WB) and reverse transcription-quantitative (RT-qPCR) assays were utilized to assess the transcription and expression of genes and proteins associated with melanogenesis. Relevant targets in the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway were verified by the addition of target inhibitors or activators. Results showed that PNEO decreased the levels of melanin and the activity of TYR in B16F10 cells. RT-qPCR and WB results showed that PNEO downregulated the expression of melanogenesis-related genes and proteins such as microphthalmia-associated transcription factor, TYR, TYR-related protein-1 and melanocortin 1 receptor, and reduced the levels of phosphorylated PKA and phosphorylated cyclic-AMP response binding protein. These results suggested that the inhibitory effect of PNEO on melanin production may be related to the cAMP/PKA pathway. Verification through the addition of target inhibitors or activators confirmed that PNEO regulates melanin synthesis and TYR activity through the cAMP/PKA signaling pathway.

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Abbreviations: AC, adenylyl cyclase; MC1R, melanocortin 1 receptor; MITF, microphthalmia-associated transcription factor; p-CREB, phosphorylated cyclic-AMP response element-binding protein; p-PKA, phosphorylated protein kinase A; PNEO, pine needle essential oil; TYR, tyrosinase; TRP-1/2, TYR-related protein-1/2; α-Ar, α-Arbutin; α-MSH, α-melanocyte-stimulating hormone; cAMP, cyclic adenosine monophosphate

Key words: PNEO, melanin synthesis, B16F10 cells, cAMP/PKA signaling pathway

Introduction

Melanocytes are a type of neural crest-derived cells that migrate to the epidermis during embryonic development (1). Upon differentiation, melanocytes spread throughout the skin and begin their main physiological function, which is the production of melanin. Melanin synthesis is a complex process that occurs within melanosomes, specialized organelles found in melanocytes responsible for pigment production and characterized by dendritic morphology (2,3). Melanin plays a crucial role in protecting the skin from UV damage (4,5). However, abnormal accumulation of melanin can lead to skin issues such as melasma, freckles and age spots (6). Numerous whitening agents on the market work by regulating melanin production to control excessive pigmentation and promote skin whitening.

One of the key regulators of melanin production is microphthalmia-associated transcription factor (MITF), which activates critical melanin synthesis-related genes such as tyrosinase (TYR), TYR-related protein (TRP)-1 and TRP-2. MITF is a common downstream target of numerous signaling pathways. The most typical way to interfere with melanin production is through the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway. When α-melanocyte-stimulating hormone (α-MSH) binds to the melanocortin 1 receptor (MC1R) expressed on melanocytes, it activates adenylyl cyclase (AC), leading to an increase in cAMP levels. cAMP then activates PKA by binding to its regulatory subunits. Phosphorylated (p-) PKA translocates to the nucleus and phosphorylates cAMP response element-binding protein (CREB), which in its phosphorylated form initiates the activation of MITF, stimulating the transcriptional expression of key enzymes related to melanin production such as TYR, TRP-1 and TRP-2 (7).

TYR is a key enzyme in melanin production, and most skin whitening agents function by inhibiting TYR activity to suppress melanin production (8). Ingredients including hydroquinone (9,10), corticosteroids (11), ascorbic acid (12,13), kojic acid (14,15) and arbutin (16) have been used as skin whitening agents to prevent or treat excessive skin pigmentation.

However, these products have drawbacks such as carcinogenicity, instability and easy degradation (10). Therefore, it is necessary to further explore natural active substances with low toxicity and effective inhibition of melanin deposition.

Plant-derived extracts or compounds were used to inhibit melanin deposition (17-19). Essential oils are extracted from various parts of plants such as leaves, flowers, roots and fruits through methods including steam distillation, cold pressing, or solvent extraction. They are composed of terpenes and other aromatic compounds (20) and possess various physiological activities such as antibacterial, insect-repellent, insecticidal, anti-inflammatory, antioxidant and anti-aging properties (21,22). Research has found that the essential oil of *Melaleuca quinquenervia* and its active components such as 1,8-cineole, α-terpineol and α-pinene can reduce TYR activity and melanin content, as well as decrease oxidative stress levels (23). Studies by Chou *et al* (24) have shown that the essential oil of *Cinnamomum cassia* and its main component cinnamaldehyde exhibit favorable anti-TYR and anti-melanin synthesis activities, along with antioxidant properties. Hsiao *et al* (25) found that the essential oil of *Calocedrus formosana* could completely inhibit melanin production at a concentration of 80 µg/ml. Further studies reported that the essential oil of *Calocedrus formosana* could significantly inhibit the expression of TRP-1, TRP-2 and MITF melanin synthesis regulatory proteins (25). Previous research has indicated that essential oils have great value in the cosmetics industry (26,27). *Pinus tabuliformis* Carrière, a member of the Pinaceae family, is an important forest product from the *Pinus* genus, with pine needles being one of the key products that can be harvested year-round due to its fast regeneration rate. Studies have reported that pine needle essential oil (PNEO) possesses various physiological activities such as antioxidant (28), anticancer (29), antibacterial (30), mental health (31) and antiviral properties (32). However, there have been no reports on the effect of PNEO from *Pinus tabuliformis* Carrière on melanin synthesis.

Based on these previous studies and our preliminary experiment, the experiment was designed as follows: A model of α-MSH-induced B16F10 melanin-overexpressing cells was used to investigate the effect of PNEO on melanin production and its molecular mechanism by using microwave assisted extraction, Gas Chromatography-Mass Spectrometry (GC-MS), reverse transcription-quantitative PCR (RT-qPCR), western blotting (WB) and other modern molecular biology techniques, including DOPA oxidation method, BCA assay kit and Cell Counting Kit-8 (CCK-8). Then the content of melanin and the activity of TYR in the cells, the levels of melanogenesis-related genes and proteins such as MITF, TYR, TRP-1 and MC1R, p-PKA and p-CREB were measured as well. The findings of the present study have important implications for the potential use of the PNEO in the cosmetics field.

Materials and methods

Reagents and equipment. B16F10 mouse melanoma cells were kindly provided by Shandong Freda Biotechnology Co., Ltd. α-Arbutin (α-Ar; CAS no: 84380-01-8, purity ≥99%) and α-α-MSH (CAS no: 171869-93-5, purity ≥97%) were obtained from Shanghai Macklin Biochemical Technology. DMEM high glucose culture medium was obtained from M&C Gene Technology. Trypsin-EDTA digestion solution was obtained from Yisheng Biotechnology. Penicillin-streptomycin solution was obtained from HyClone; Cytiva. Fetal bovine serum (FBS) was obtained from Wuhan Pricella Biotechnology Co., Ltd. The CCK-8 was purchased from Biosharp Life Sciences. L-DOPA (CAS: 59-92-7, purity ≥98%) was sourced from Beijing Solarbio Science & Technology Co., Ltd. H89 (PKA inhibitor), IBMX (cAMP activator) and Forskolin (AC activator) were acquired from Beyotime Institute of Biotechnology. RNA extraction reagent, chloroform substitute, RNA dissolution solution, beta-actin (cat. no. GB11001) and HRB-conjugated Goat Anti-Rabbit IgG H&L secondary antibodies (cat. no. GB23303), primary antibody diluent (cat. no. G3337) were obtained from Wuhan Servicebio Technology Co., Ltd. RIPA buffer (purity ≥98%) was sourced from Beijing Solarbio Science & Technology Co., Ltd.

The GC-MS system 8890-5977B was purchased by Agilent. The LS-C0105 CO_2 incubator was purchased by NuAire Lab Equipment (https://www.nuaire.com/). The SW-CJ-2D type ultra-clean workbench was procured by Suzhou Hengda Purification Equipment. The HVE-50 high-pressure steam sterilizer pot was obtained from Xinhua Medical Equipment Co., Ltd. and the CKX53 inverted microscope was purchased from Olympus Corporation.

Sample processing. The PNEO was prepared in the Natural Products Research Center of Shandong Normal University. A total of 20 g of pine needle powder from *Pinus tabuliformis* Carrière was dissolved in distilled water, and the PNEO was extracted by using microwave-assisted extraction technology and stored at 4˚C (33).

PNEO analysis by GC-MS. GC-MS was used to analyze the constituent of PNEO. The analysis employed an Agilent HP-5MS column (30 mm x 0.25 mm x 0.25 µm). The temperature program of the column was initiated at 60˚C, maintained for 1 min, ramped up at a rate of 8˚C/min to 140˚C and held for 2 min the temperature was increased at 2˚C/min to 240˚C and held for 2 min, followed by a further ramp up at 8˚C/min to 280˚C, where it was held for 5 min. Helium (purity, ≥99.999%) was used as the carrier gas at a flow rate of 1.0 ml/min. The inlet temperature was set at 260˚C, and a split injection mode with a split ratio of 10:1 was employed, with an injection volume of 1 µl.

Mass spectrometry analysis was carried out by using instrument utilized an Electron Impact (EI) ionization mode with a voltage of 70 eV, a source and transfer line temperatures of 280˚C. The detection mode operated in full scan (SCAN) mode, nitrogen (N_2) (purity, ≥99.999%) was used as the carrier gas. A solvent delay of 3 min was implemented to optimize the analysis process.

Cell culture and grouping. B16F10 melanoma cells were cultivated in DMEM medium containing 10% FBS. The cells were maintained in a humidified 5% CO_2 incubator at 37˚C and were sub-cultured every 2-3 days to maintain logarithmic growth for subsequent experimentation. Experimental groups included: i) A control group; ii) PNEO treatment group (300 nM α-MSH + 12.5, 25, 50, 100, 200,

400, 800 μg/ml PNEO); iii) α-MSH model group (300 nM α-MSH); and iv) a positive control group (300 nM α-MSH + 100 μg/ml α-Ar).

CCK-8 cell viability assessment. The cell viability was determined by using the CCK-8 method. B16F10 cells were seeded in a 96-well plate at a density of 5x10^4 cells/ml. Following a 24-h incubation period, cells were categorized based on Section 2.2.2 and cultured for an additional 24 or 48 h. A 10% CCK-8 working solution was prepared in DMEM medium, replacing the original medium with 100 μl of the working solution per well. Subsequently, cells were further incubated at 37˚C for 1 h before measuring the optical density (OD) at 450 nm using a microplate reader. Cell viability was calculated as follows: $(A_1\text{-}A)/(A_0\text{-}A)$ x100%. A_1 represents the OD value of the group treated with drugs, including cells, CCK-8 solution and drug solution; A denotes the OD value of the control group with medium and CCK-8 solution but no cells; and A_0 signifies the OD value of the group containing cells, medium and CCK-8 solution.

BCA protein assay kit for protein concentration determination. The protein concentration was determined using the BCA Protein Assay Kit following the manufacturer's protocol.

Assay of melanin content. Melanin content was quantified using the NaOH lysis method. B16F10 cells were seeded in 6-well plates at a density of 1.25x10^5 cells/ml per well, with 2 ml of cell suspension added to each well and then incubated for 24 h. Subsequently, abandoning the original culture medium, 2 ml of fresh medium was added to each well according to the aforementioned grouping. Following an additional 48-h static incubation, cells were washed twice with PBS, harvested by scraping, and centrifuged at 13,201 x g for 5 min at 4˚C. The resulting supernatant was discarded, and 300 μl of 1 M NaOH solution, containing 10% DMSO, was added to each centrifuge tube. After thorough mixing and incubation at 80˚C to solubilize the melanin, vigorous vortex followed. B16F10 lysate containing melanin was then dispensed into a 96-well plate (100 μl per well, with 3 replicates per group), and the absorbance at 405 nm was measured for each well using a microplate reader to determine the relative melanin content. Relative melanin content was calculated as follows: A_1/A_0 x 100%. A_1 represents the OD value of the experimental group, and A_0 represents the OD value of the control group.

Assay of TYR activity. The TYR activity within B16F10 cells was assayed in terms of DOPA oxidase activity. The plating and drug administration processes were carried out as aforementioned. After continuous static culture for 48 h, the cells were washed twice with PBS. Then, 500 μl of 1% RIPA solution was added to each well, and the plate was quickly placed in a -80˚C refrigerator for freezing. After being taken out, it was placed at room temperature to thaw so that the cells were ruptured. This freeze-thaw process was repeated several times. Then the cells were collected and centrifuged at 4˚C, 9,167 x g for 5 min. The supernatant was reserved in an ice bath. In a 96-well plate, 50 μl of the supernatant was added, followed by 50 μl of 1 mM L-DOPA solution. After reacting at 37˚C for 1 h, the absorbance was measured at 492 nm. The TYR activity was preliminarily calculated according to the protein concentration, and then the relative activity was compared. Relative TYR activity was calculated as follows: C_1/C_0 x 100% (3). C_1 represents TYR value of the experimental group, and C_0 represents TYR value of the control group.

RT-qPCR detection of melanin biosynthesis-related gene expression. RNA Extraction Solution (cat. no. G3013) was sourced from Wuhan Seavil Biotechnology Co., Ltd. Reverse transcription (RT) kit (cat. no. G3337) was provided by Wuhan Saiwell Biotechnology Co., Ltd. Total RNA from B16F10 cells treated with PNEO at a mass concentration of 50 μg/ml and the positive control group α-Ar for 48 h was subjected to reverse transcription on a regular PCR instrument. The cDNA first-strand product obtained from reverse transcription (temperature protocol: 25˚C for 5 min, then 42˚C for 30 min, final 85˚C for 5 sec) served as the template, with various reaction components sequentially added to the tube for amplification on a fluorescent quantitative PCR instrument. SYBR green (obtained from Wuhan Saiwell Biotechnology Co., Ltd.) was used as fluorophore for qPCR. Thermocycling conditions were as follows: Stage 1, 95˚C, 30 sec pre-denaturing; Stage 2, 95˚C, 15 sec denaturation; 60˚C, 30 sec annealing/elongation; Stage 3, 65-95˚C. The expression levels of the *Tyr, Trp-1, Trp-2, Mitf* and *Mc1r* genes were analyzed using the $2^{-\Delta\Delta Cq}$ method. Primer sequences are included in Table I.

Detection of melanin biosynthesis-related protein expression. The protein expression levels associated with melanin biosynthesis in B16F10 cells were assessed through western blot (WB) analysis. B16F10 cells were cultured for 24 h based on distinct groupings, exposed to PNEO at a concentration of 50 μg/ml for 48 h, rinsed twice with PBS, desiccated, harvested, lysed, and the resulting lysates were transferred into 1.5-ml centrifuge tubes containing an appropriate volume of RIPA lysis buffer. Cell lysis was carried out on ice for 30 min to ensure complete cellular disruption. Subsequent steps included centrifugation at 4˚C, 12,000-16,000 x g for 10 min, determination of protein concentration, denaturation, SDS-PAGE electrophoresis (10% acrylamide and 25-30 μg protein loaded per lane), PVDF membrane transfer, incubation at room temperature for 30 min for blocking (5% skim milk), addition of primary antibody, and overnight agitation at 4˚C. The membrane underwent three 5-min washes with TBST (0.1% Tween-20), followed by incubation at room temperature for 30 min with a secondary antibody diluted in TBST at a ratio of 1:5,000. After three additional rapid 5-min washes with TBST, the membrane was exposed, and the original image was preserved for subsequent data analysis. An immunoblot image analysis software based on artificial intelligence learning, launched by Wuhan Servicebio Technology Co., Ltd., was used.

Statistical analysis. Each experiment was replicated three times, and the results are presented as the mean ± standard deviation (X ± SD). Data analysis was conducted using GraphPad Prism 9.0.0 software (Dotmatics), employing one-way analysis of variance for comparisons among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Gene name	Primer sequence (5'-3')
Tyr	Sense: TAACTTACTCAGCCCAGCATCC
	Antisense: ATAGTGGTCCCTCAGGTGTTCC
Trp-1	Sense: TTCGTTGGAGCTGTGATTGTTG
	Antisense: AGGAATAATGTTGAAAGGTGGGG
Trp-2	Sense: AGAAACAACCCTTCCACAGATGC
	Antisense: AAGCTCCCAGGATTCCAATGAC
Mitf	Sense: GCCCTATGGCTATGCTCACTCTT
	Antisense: TGTTCATACCTGGGCACTCACTC
Mc1r	Sense: CTCATTGACGTGCTCATCTGTGG
	Antisense: TGCTTGTAGTAGGTGATAAAGAGGGT
Creb	Sense: TGGCTAACAATGGTACGGATGG
	Antisense: GTGCTGTGCGGATCTGGTATGT
Crtc1	Sense: AGAAGATCGCACTGCACAACCA
	Antisense: CCACGCTGCTGCTTCCAAT
Prkaca	Sense: ATCGTCCTGACCTTTGAGTATCTG
	Antisense: AACCGAAGTCTGTCACCTGAATAT
GAPDH	Sense: CCTCGTCCCGTAGACAAAATG
	Antisense: TGAGGTCAATGAAGGGGTCGT

Tyr, tyrosinase; Trp, Tyr-related protein; MITF, microphthalmia-associated transcription factor; MC1R, melanocortin 1 receptor; CREB, cyclic-AMP response element-binding protein; CRTC1, CREB-regulated transcription coactivator 1.

Results

Analysis of PNEO by GC-MS. The total ion chromatogram of the PNEO obtained by microwave-assisted extraction is shown in Fig. S1 (33), and a summary of its components and relative peak areas is presented in Table SI. By conducting GC-MS analysis of the PNEO extracted using the microwave-assisted method, a total of 332 substances were identified. Peaks with matching degree exceeding 80% were considered as candidate compounds, and after qualitative comparison by retrieving the NIST spectral library (https://webbook.nist.gov/chemistry/), 103 chemical compounds were identified, accounting for 47.39% of the total composition. The relative percentage content of each component in the PNEO was calculated using area normalization. The results showed that the PNEO mainly contained alcohols (11.01%), hydrocarbons (11.04%), esters (9.3%) and terpenes (2.997%). These included Thunbergol (PubChem CID: 5363523; 3.938%), Verticillol (PubChem CID: 5377475; 3.597%), Hentriacontane (PubChem CID: 12410; 2.189%), α-Terpinyl acetate (PubChem CID: 111037; 1.901%), Methyl dehydroabietate (PubChem CID: 14697; 1.842%), α-cadinene (PubChem CID: 12306048; 1.534%), Isopimara-8 (PubChem CID; 13783133) (14), 15-dien-19-saeure-methylester (PubChem CID: 13710744; 1.517%), Cembrene (PubChem CID: 6430770; 1.139%) and α-Terpinene (PubChem CID: 7462; 0.825%), among others.

Effect of PNEO on B16F10 cell viability. The CCK-8 results are demonstrated in Fig. 1A. Treatment of cells with different concentrations of PNEO for 24 h showed a significant impact on cell viability when the concentration exceeded 400 μg/ml. At concentrations <400 μg/ml, cell morphology was normal, and the cell viability was >85%, with no significant difference compared with the control group (P>0.05). After 48 h of treatment, cell viability decreased significantly with increasing concentration of PNEO. At concentrations <50 μg/ml, PNEO did not affect cell viability (P>0.05). However, when the PNEO concentration was 100 μg/ml, the cell viability significantly decreased to 81.89±3.27% (P<0.01). Subsequently, PNEO concentrations of 12.5, 25 and 50 μg/ml were selected for further experiments.

Effect of PNEO on melanin content in B16F10 cells. After 48 h of intervention with 12.5, 25 and 50 μg/ml of PNEO, the melanin content in B16F10 cells was measured. As can be observed in Fig. 1B, when the cells were stimulated by α-MSH, the relative melanin content was 156.25±1.70%, indicating that the high melanin expression cell model was successfully developed. PNEO dose-dependently inhibited melanin content, with melanin inhibition rates of 24.81±1.31%, 31.63±1.31% and 54.36±0.67% in the low, medium and high dose groups, respectively. At 50 μg/ml, PNEO was able to achieve inhibition effects similar to the α-Ar positive control.

Effect of PNEO on TYR activity in B16F10 cells. The results of TYR activity measurement are demonstrated in Fig. 1C. Compared with the control group, the TYR activity in the α-MSH group was 185.22±1.58% (P<0.0001), indicating the success of the model. Different mass concentrations of PNEO and the α-Ar positive control group inhibited TYR activity in B16F10 cells, showing significant differences compared with the α-MSH model group. In the α-Ar positive control group, the

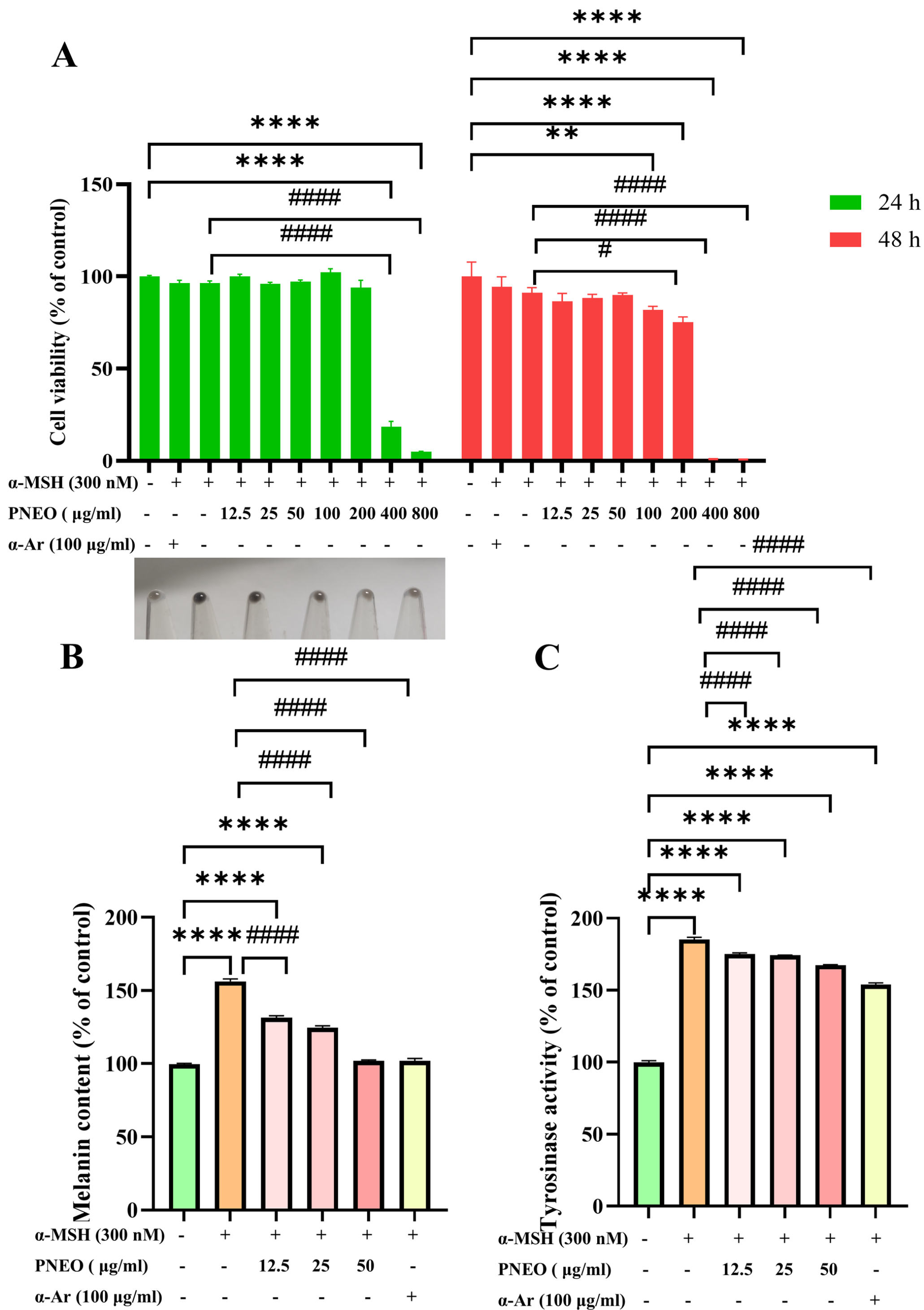

Figure 1. Effect of PNEO on cell viability and melanin production and tyrosinase activity in B16F10 cells (n=3). (A) The activity of B16F10 cells. (B) Melanin content. (C) Tyrosinase activity. PNEO, pine needle essential oil; α-Ar, α-Arbutin; α-MSH, α-melanocyte-stimulating hormone. **P<0.01 and ****P<0.0001; #P<0.05 and ####P<0.0001.

inhibition rate of TYR in B16F10 cells reached 31.36±17.25%, while PNEO at a mass concentration of 50 μg/ml exhibited the strongest inhibition of TYR activity, with an inhibition rate of 17.77±8.57%. This suggests that PNEO could effectively inhibit TYR activity in α-MSH-induced B16F10 cells.

Effect of PNEO on the expression of melanogenesis-related genes. MITF, TYR, TRP-1 and TRP-2 are important signaling molecules in the process of melanogenesis. Based on the results of melanin content and TYR activity, 50 μg/ml of PNEO achieved the optimal inhibitory effect. Therefore, the concentration of PNEO was set at 50 μg/ml in RT-qPCR and WB detection. The gene expression levels of *Tyr*, *Trp-1*, *Trp-2*, *Mitf* and *Mc1r* are revealed in Fig. 2. After treatment with PNEO for 48 h, the expression of *Tyr*, *Trp-1*, *Mitf* and *Mc1r* genes in B16F10 cells was significantly inhibited compared with the α-MSH model group. However, the expression level of *Trp-2,* which

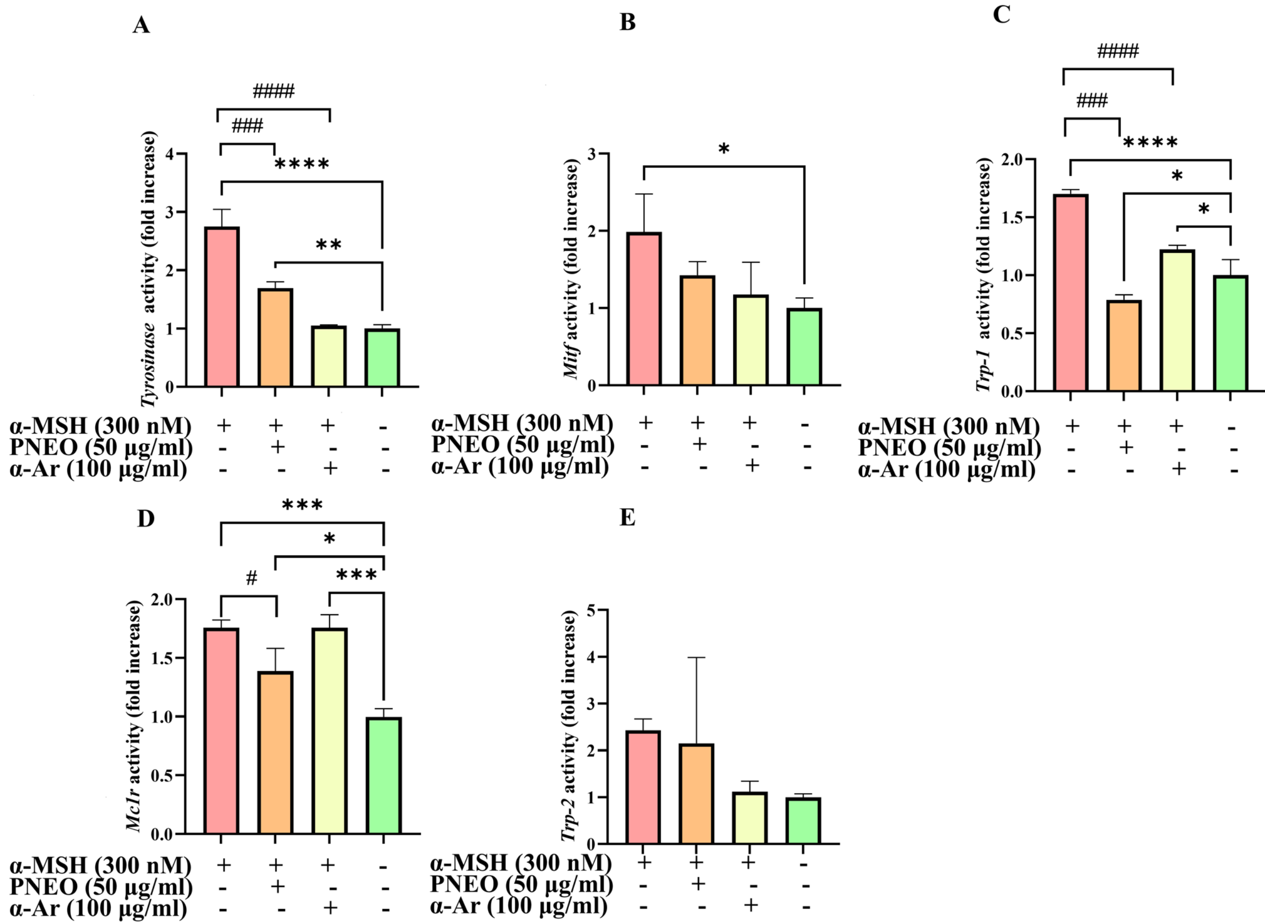

Figure 2. Impact of pine needle essential oil on gene expression in B16F10 cells (n=3). (A) Tyrosinase. (B) *Mitf*. (C) *Trp-1*. (D) *Mc1r*. (E) *Trp-2*. PNEO, pine needle essential oil; α-Ar, α-Arbutin; α-MSH, α-melanocyte-stimulating hormone; MC1R, melanocortin 1 receptor; MITF, microphthalmia-associated transcription factor; TRP, tyrosinase-related protein. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; #P<0.05, ###P<0.001 and ####P<0.0001.

is related to melanin production, showed no significant impact.

Effect of PNEO on the expression of melanin synthesis-related proteins. Through WB experiments, the effect of PNEO on the proteins MITF, TYR, TRP-1 and TRP-2, as well as the phosphorylation levels of proteins in the cAMP/PKA signaling pathway was examined to elucidate the mechanism of action by which PNEO inhibits melanin production in B16F10 cells (Fig. 3). Treatment of B16F10 cells with 50 μg/ml PNEO for 48 h resulted in decreased expression of TYR, TRP-1, TRP-2, MITF and MC1R proteins.

Verification of the effect of PNEO on the cAMP/PKA signaling pathway. The CREB molecule is one of the phosphorylation substrates of various protein kinases. The expression level of p-CREB in the nucleus can indirectly reflect the activity of PKA in the cytoplasm. CREB-regulated transcription coactivator 1 (CRTC1) is dephosphorylated and enters the nucleus, forming a CREB/CRTC1 heterodimer in the nucleus, which enhances the transcription levels on the MITF-M promoter. To verify whether PNEO acts on the cAMP/PKA signaling pathway, the results are shown in Fig. 4. Treatment with PNEO resulted in decreased expression of *Crtc1* and *Prkaca*. After treatment of B16F10 cells with PNEO for 48 h, the protein levels of p-CREB and p-PKA were significantly inhibited (Fig. 5). It was hypothesized that the cAMP/PKA signaling pathway is involved in the process by which PNEO inhibits melanin production.

To further investigate the molecular mechanism underlying the inhibitory effect of PNEO on melanin production, the effects of the PKA inhibitor H89, the cAMP activator IBMX, and the AC activator Forskolin on the signaling pathways related to melanin production were studied in the context of PNEO-mediated melanin inhibition. The results, as shown in Fig. 6A, demonstrate that the addition of PNEO or the H89 inhibitor significantly reduced melanin content, with a synergistic effect observed between the two, resulting in a melanin content of 88.93±1.07%. In Fig. 6B, the results of cellular TYR activity identified that treatment with α-MSH significantly increased TYR activity, while the addition of PNEO or H89 inhibitors decreased TYR activity to 83.55±1.44% and 77.41±0.24%, respectively. Additionally, treatment with PNEO in combination with IBMX or Forskolin activators resulted in increased melanin content (Fig. 6C) and TYR activity (Fig. 6D), with Forskolin activator showing a more significant promotion effect, reaching a TYR activity of 235.18±4.50%. In the groups treated with α-MSH, IBMX, or Forskolin activators in combination with PNEO, both melanin content and TYR activity decreased. These findings validate that PNEO regulates melanin production through the cAMP/PKA signaling pathway.

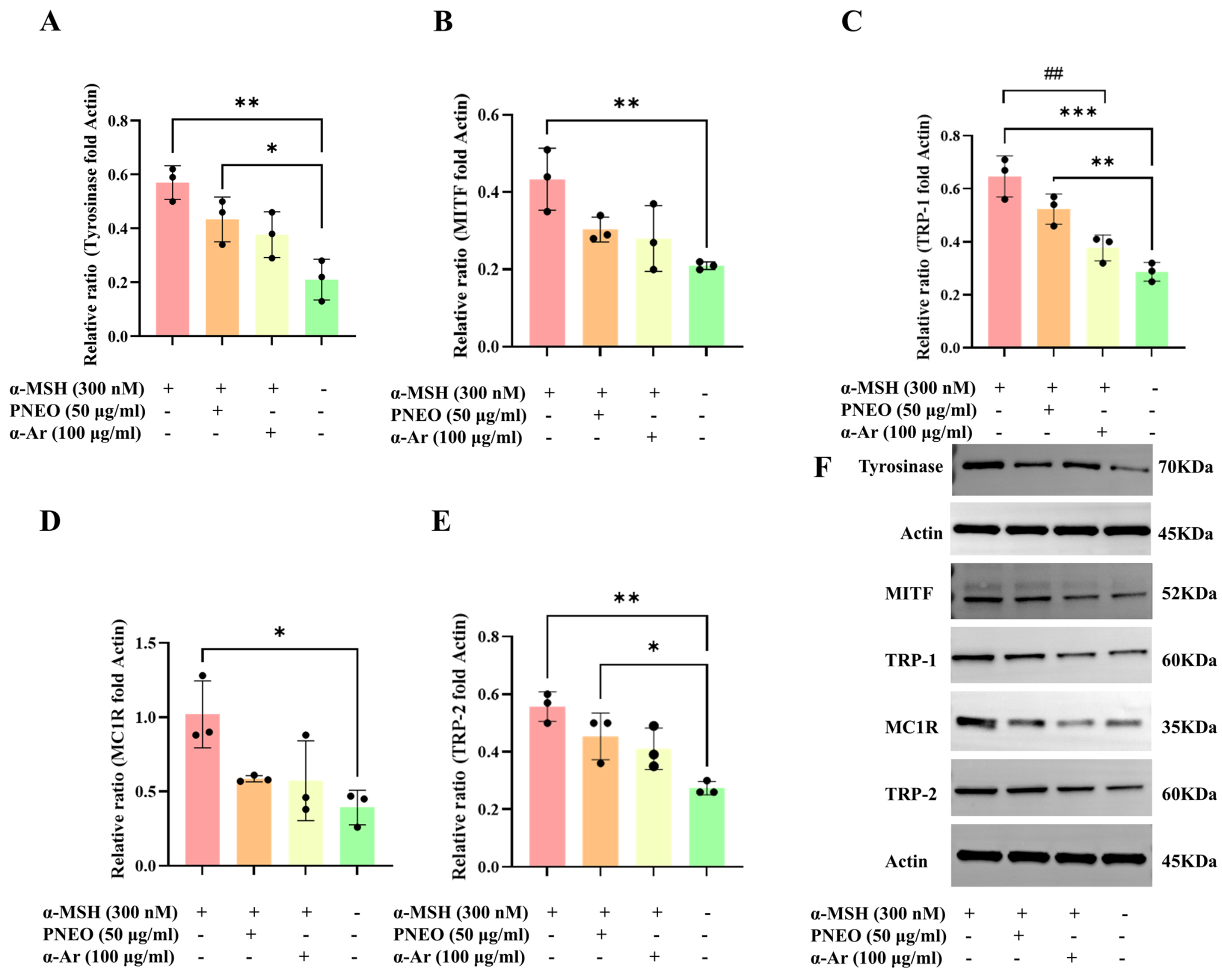

Figure 3. Results of western blot analysis (n=3). (A) Tyrosinase. (B) Mitf. (C) Trp-1. (D) Mc1r. (E) Trp-2. (F) Western blot analysis of the aforementioned proteins. PNEO, pine needle essential oil; α-Ar, α-Arbutin; α-MSH, α-melanocyte-stimulating hormone; MC1R, melanocortin 1 receptor; MITF, microphthalmia-associated transcription factor; TRP, tyrosinase-related protein. *P<0.05, **P<0.01 and ***P<0.001; $^{\#\#}$P<0.01.

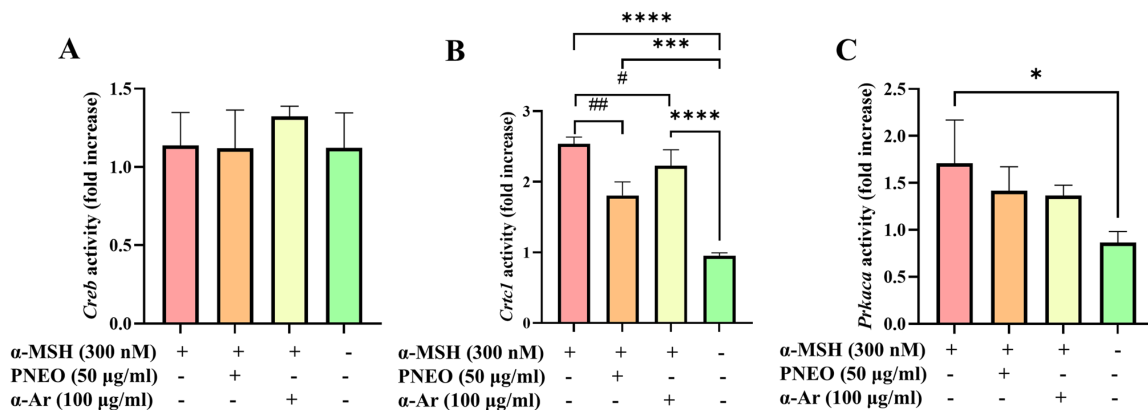

Figure 4. Effect of PNEO on the expression of genes related to cAMP-PKA signaling pathway (n=3). (A) *Creb*. (B) *Crtc1*. (C) *Prkaca*. PNEO, pine needle essential oil; α-Ar, α-Arbutin; α-MSH, α-melanocyte-stimulating hormone; PKA, protein kinase A; CREB, cyclic-AMP response element-binding protein; CRTC1, CREB-regulated transcription coactivator 1. *P<0.05, ***P<0.001 and ****P<0.0001; $^{\#}$P<0.05 and $^{\#\#}$P<0.01.

Discussion

The present study utilized microwave-assisted extraction to extract essential oil from *Pinus tabulatus* pine needles. Microwave energy generates heat that causes plant cell wall rupture, facilitating rapid dissolution of the extract (34), thereby reducing extraction time compared with traditional steam distillation and offering advantages such as energy savings and reduced production costs (35). Properly reducing the extraction time with microwave-assisted extraction lowers the likelihood of thermal degradation of the extract (36). Additionally, research indicates that plant essential oils

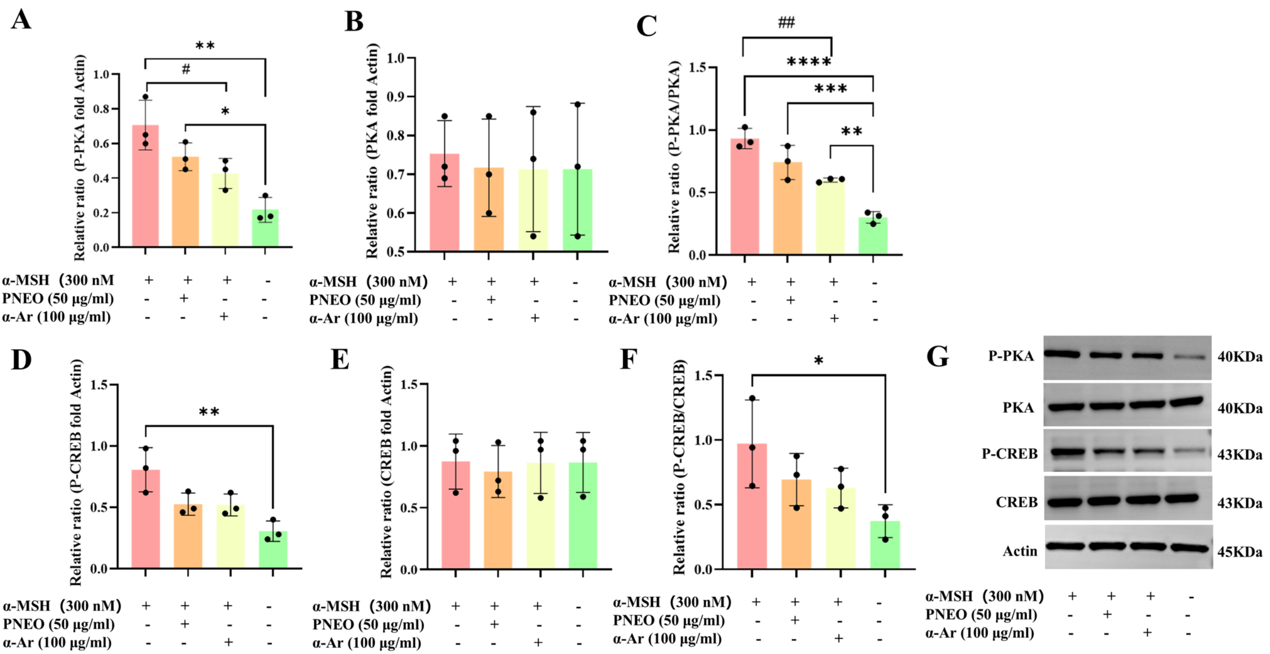

Figure 5. Effect of PNEO on the phosphorylation level of PKA and CREB proteins in B16F10 cells (n=3). (A) P-PKA. (B) PKA. (C) Ratio of P-PKA to PKA protein relative quantity. (D) P-CREB. (E) CREB. (F) Ratio of P-CREB to CREB protein. (G) Western blot analysis of the aforementioned proteins. PNEO, pine needle essential oil; CREB, cyclic-AMP response element-binding protein; p-, phosphorylated; α-Ar, α-Arbutin; α-MSH, α-melanocyte-stimulating hormone. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; #P<0.05 and ##P<0.01.

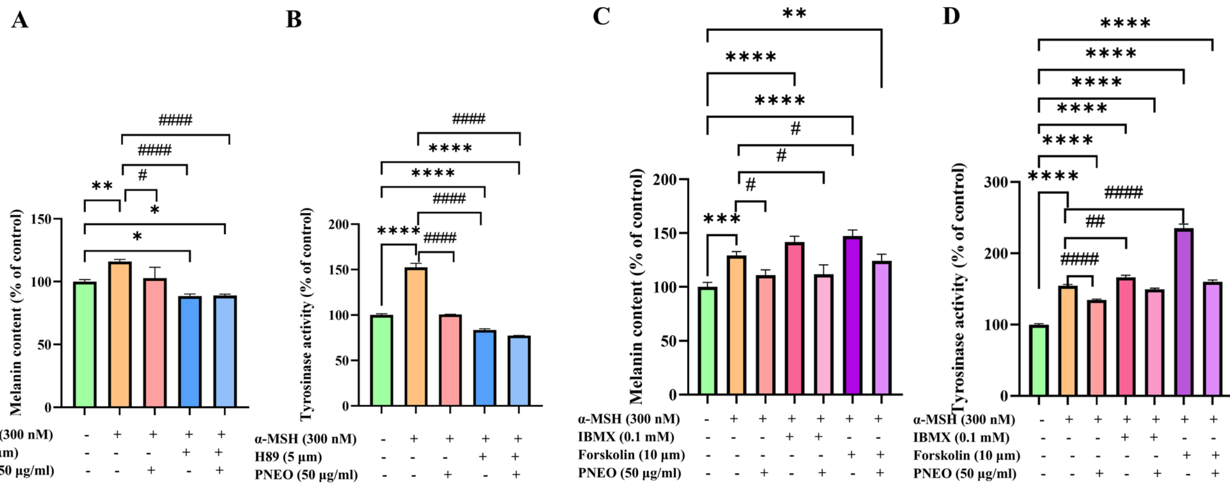

Figure 6. Effects of H89, IBMX and Forskolin on melanin content and tyrosinase activity in B16F10 cells treated with PNEO (n=3). (A) Melanin content treated with H89. (B) Tyrosinase activity treated with H89. (C) Melanin content treated with IBMX and Forskolin. (D) Tyrosinase activity treated with IBMX and Forskolin. PNEO, pine needle essential oil; α-MSH, α-melanocyte-stimulating hormone. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; #P<0.05, ##P<0.01 and ####P<.0001.

extracted using microwave assistance exhibit strong antioxidant capabilities and have a longer shelf life (37,38). In the present study, the effects of microwave-assisted extraction of PNEO on the viability of B16F10 cells were initially tested. The results demonstrated that PNEO had no significant toxic effects on cells at concentrations of 12.5-50 μg/ml. Using α-Ar as a positive control group, the impact of PNEO on melanin content and TYR activity within B16F10 cells was evaluated. The findings revealed that different concentrations of PNEO exhibited significant inhibitory effects on melanin content and TYR activity within the cells. At a concentration of 50 μg/ml, PNEO achieved a similar inhibitory effect on melanin content as α-Ar and significantly suppressed TYR activity. Studies have reported that inhibiting TYR activity is an effective approach to inhibiting melanin formation (39). Previous studies reported that the anti-melanin activity of plant essential oils was mainly attributed to terpenes such as α-pinene, limonene, β-laurene and β-pinene (23,40,41). Additionally, minor components in the oil demonstrate favorable physiological activity and exhibit superior effects in inhibiting melanin formation (42). This suggests that the whitening potential of PNEO may be attributed to the actions of various terpenes present in the oil.

When the skin is exposed to ultraviolet light, keratinocytes produce α-MSH, which in turn induces the MC1R signaling

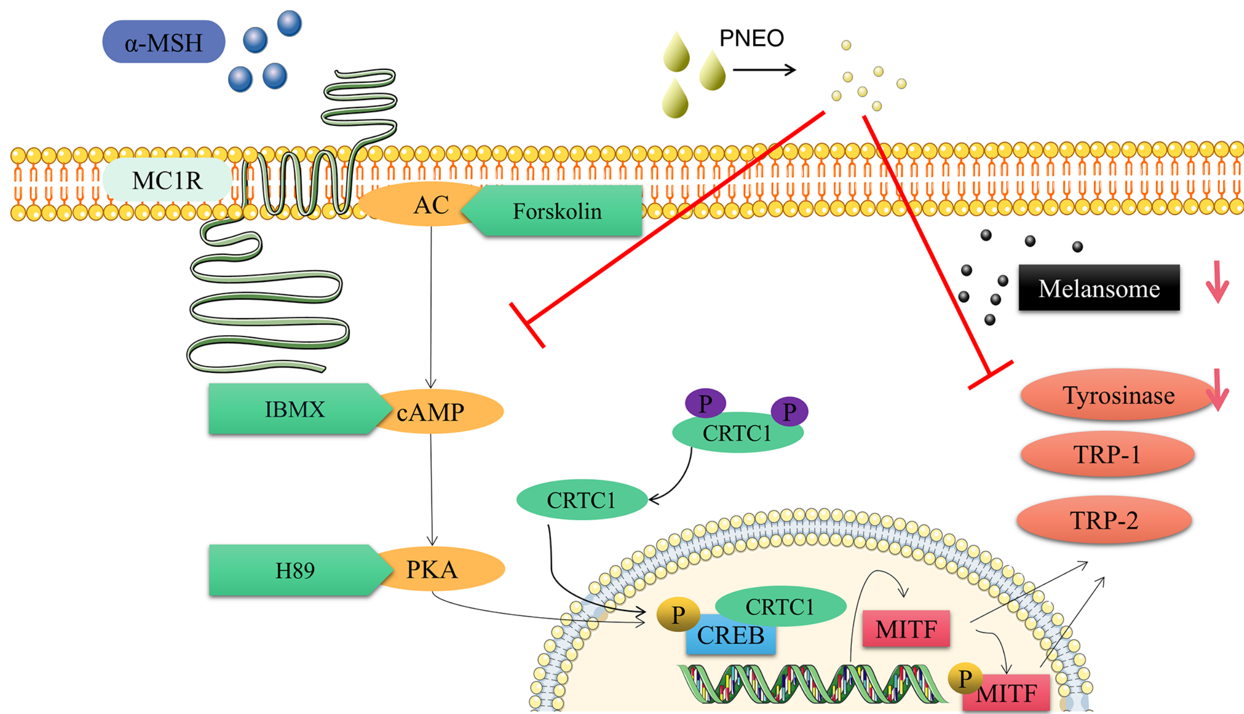

Figure 7. Regulatory mechanism of melanin synthesis in B16F10 cells by PNEO. The red arrows indicate decrease expression levels, and the red T bars indicate inhibition. PNEO, pine needle essential oil; α-MSH, α-melanocyte-stimulating hormone; PKA, protein kinase A; MITF, microphthalmia-associated transcription factor; MC1R, melanocortin 1 receptor; CREB, cyclic-AMP response element-binding protein; CRTC1, CREB-regulated transcription coactivator; AC, adenylyl cyclase; TRP, tyrosinase-related protein.

pathway in melanocytes (43,44). The MC1R signaling pathway is crucial for melanin formation. α-MSH activation of the MC1R pathway leads to activation of the cAMP signaling pathway, which subsequently stimulates AC and cAMP production downstream of α-MSH-induced MC1R activation, activating PKA and CREB protein (45). CREB, when phosphorylated, activates and induces the expression of *Mitf*, which in turn activates the transcription of melanin synthesis-related genes: *Mitf*, *Trp-1*, *Trp-2* and *Tyr*. Additionally, *Mitf* is essential for the development of melanocytes, melanin formation and long-term cell survival (46). Theasinensin A in tea significantly reduces the mRNA expression of *Tyr*, *Trp-1* and *Trp-2*. It also inhibits the increase in TYR and MITF protein levels during α-MSH exposure, as well as inhibits the phosphorylation of CREB and PKA, thereby reducing melanin synthesis through the cAMP signaling pathway (47). To elucidate the mechanism by which PNEO inhibits melanin production and to identify the signaling pathways regulated by PNEO during the inhibition of melanin production, treatment with PNEO reduced the expression levels of the genes *Tyr*, *Trp-1*, *Mitf*, *Mc1r*, *Crtc1* and *Prkaca* in B16F10 cells, which are important genes in the melanin production process. Among them, TRP-1 is a 75-kDa protein synthesized in the endoplasmic reticulum, transported through the Golgi apparatus, and transferred to melanosomes (48). TRP-1 enhances TYR activity by forming a stable complex to increase its enzymatic activity and is also involved in the proliferation and morphology of melanocytes (49). Research has shown that diacetyl-caffeic acid cyclohexyl ester can block the nuclear entry of CRTC1 in melanocytes, inhibit the formation of the CREB/CRTC1 heterodimer, decrease the transcription levels on the MITF-M promoter, thus reducing melanin production. CRTC1 is potentially a therapeutic target for pigmentary disorders including melasma, freckles and senile lentigines (50).

Unlike previous studies, PNEO extraction was found to have no significant impact on the expression of the *Trp-2* gene. Various pathways are known to reduce melanin production, with past research indicating that intracellular melanin synthesis is primarily influenced by TYR activity. Inhibition of TYR activity significantly weakens melanin synthesis in melanocytes. The findings of this study suggest that PNEO can reduce the levels of MITF, TYR, TRP-1, TRP-2, and MC1R proteins, as well as decrease the phosphorylation levels of molecules related to the cAMP/PKA signaling pathway. Phosphorylated CREB upregulates MITF levels. Treatment with PNEO decreases the levels of the MC1R receptor, leading to a significant reduction in the levels of downstream PKA and CREB phosphorylation in the cAMP signaling pathway, inhibiting MITF activation and expression. This results in the lowered levels of proteins associated with melanin production (TYR, TRP-1, TRP-2), ultimately suppressing melanin production through the cAMP/PKA/CREB signaling pathway.

Similar research findings have been reported previously. For instance, Seo *et al* (51) discovered in their study on the anti-melanogenesis effects of *Leathesia difformis* extracts that an increase in α-MSH levels leads to the binding of the MC1R receptor on melanocyte cell membranes, causing an elevation in cAMP and activation of the downstream signaling molecule PKA. This, in turn, increases *Mitf* expression via CREB activation. Seaweed extracts were found to downregulate the expression of genes related to melanin synthesis, reducing p-CREB levels, indicating that seaweed extracts may

inhibit melanin production through the cAMP/PKA/CREB pathway (51).

The effects of plant extracts on the mechanism of melanin production have been previously investigated. The root extract of *Astragalus membranaceus* inhibits the downregulation of MITF, mediated by cAMP response, CREB and p38 MAPK kinase. The PKA inhibitor H89 and the p38 inhibitor SB203580 have validated that formononetin inhibits melanin synthesis and TYR activity by regulating the PKA/CREB and p38 MAPK signaling pathways (52).

To verify the effect of PNEO on the targets of melanin production signaling pathways, the present study used PNEO treatment while adding the PKA inhibitor H89. The levels of melanin production and TYR activity were further reduced. When cAMP activator IBMX and AC activator Forskolin were added, the levels of melanin production and TYR activity increased. However, adding PNEO weakened this increasing trend. It was validated that PNEO can regulate melanin production and TYR activity through the cAMP/PKA signaling pathway. The results of the present study provide a certain basis for the utilization of pine needle resources. Subsequently, the authors will analyze the effect of the screened components on melanin production through B16F10 cell experiments and explore the signal pathways involved in their inhibitory effect on melanin production. In future studies, a major chemical component in PNEO will be selected and more in-depth research shall be carried out, which will be helpful for the exploration of the pharmacological mechanism of the essential oil.

In conclusion, the present results indicate that PNEO inhibits melanin production through the cAMP/PKA signaling pathway. Mechanistically, PNEO inhibits *Mitf* expression in B16F10 cells and downstream enzymes such as TYR, TRP-1 and TRP-2 by modulating PKA and CREB protein phosphorylation to suppress melanin synthesis (Fig. 7). This will have important implications for the potential use of the PNEO in the cosmetics field.

PNEO downregulates the expression of *Tyr*, *Trp-1*, *Crtc1*, *Mc1r* and *Mitf* genes, as well as TYR, TRP-1, TRP-2, MC1R and MITF proteins in B16F10 cells. It also diminishes the phosphorylation levels of molecules linked to the cAMP/PKA signaling pathway, thus lowering TYR activity and inhibiting melanin synthesis. PNEO or the H89 inhibitor notably reduces melanin content, whereas IBMX or Forskolin activators sustainably elevate melanin content and TYR activity. However, co-treatment with PNEO decreases melanin content and TYR activity. These results suggest that PNEO inhibits melanogenesis via the cAMP/PKA signaling pathway.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SL conducted investigation and formal analysis, and wrote the original draft. BS and GL validated and curated data. LS conducted software analysis and data visualization. QW developed the methodology, acquired funding and wrote, reviewed and edited the manuscript. YG conceptualized and supervised the study, performed project administration, and wrote, reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. YG and SL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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