Fitoterapia 145 (2020) 104634

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

# Anti-melanogenic effects of epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG) and gallocatechin-3-gallate (GCG) via down-regulation of cAMP/CREB /MITF signaling pathway in B16F10 melanoma cells

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ARTICLE INFO

*Keywords:*
Tea catechins
B16F10 cells
Melanin
Melanogenesis
Tyrosinase

ABSTRACT

Tea catechins, the main bioactive polyphenols in green tea, are well known for their health promoting effects. Previous studies have shown that gallocatechin-3-gallate (GCG), epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG) exerted strong inhibitory effects on mushroom tyrosinase activity in vitro, whilst EGCG inhibited melanogenesis in vivo, yet the underlying mechanisms are not entirely clear. In this study, we (i) evaluated and compared the inhibitory effects of the main tea catechins (GCG, EGCG, and ECG) on melanogenesis in B16F10 melanoma cells, and (ii) explain the underlying mechanisms. The results showed that the tea catechins significantly suppressed tyrosinase activity and melanin synthesis in B16F10 cells, where the effects of ECG > EGCG > GCG. Interestingly, the inhibitory effects of the catechins were stronger than those of arbutin (AT), a well-known depigmenting agent. Moreover, GCG, EGCG, and ECG regulated the melanogenesis of B16F10 cells through the cAMP/CREB/MITF pathway. These results revealed catechins could be used as anti-melanogenic agents to protect cells from abnormal melanogenesis.

## 1. Introduction

Melanin is a multifunctional biopolymer. It is synthesized in the melanosomes of melanocytes located at the basal layer of the epidermis and subsequently transferred to surrounding keratinocytes. Its synthesis is regulated by a large number of melanogenic stimuli, including ultraviolet B and cyclic adenosine monophosphate (cAMP) elevating agents such as α-melanocyte stimulating hormone (α-MSH) and estrogen [1–3]. Melanin has many crucial physiological functions, including protection of skin from ultraviolet radiation-induced damage [4]. However, overproduction and accumulation of melanin can result in the darkening of skin and/or skin diseases, including freckles, chloasma, pigmentation, post-inflammatory melanosis, and solar lentigo [5,6].

Melanin exists in two basic forms in human skin, that is, brown-to-black eumelanin and yellow-to-reddish pheomelanin, which are synthesized from tyrosine by a common biosynthetic pathway [7]. In the first two steps of melanogenesis, tyrosinase (the rate-limiting enzyme) catalyzes the hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to L-dopaquinone. On one hand, L-dopaquinone can be converted to cysteinyl-DOPA, which is further oxidized to pheomelanin. On the other hand, L-dopaquinone can be converted to L-dopachrome, and then dopachrome tautomerase (DCT; TRP-2) isomerizes L-dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Subsequently, TRP-1 oxidizes DHICA to indole-5,6-quinone-2-carboxylic acid, which is eventually converted into eumelanin [8–10]. Therefore, inhibition of the activities of an enzymatic cascade consisting of several key enzymes, such as tyrosinase, TRP-1, and TRP-2, is considered as an important method in the development of whitening cosmetics or dermatological drugs.

It is well established that signaling pathways such as cAMP/cAMP-responsive element-binding protein (CREB), Wnt/β-catenin, and SCF/c-kit are involved in the synthesis of melanin in melanocytes [11–13]. The cAMP-mediated CREB signaling pathway is the predominant cascade regulating melanin production. Microphthalmia-associated transcription factor (MITF) is the most important molecular target among these pathways, and alterations in MITF expression are strongly associated with abnormal skin and hair pigmentation [14]. MITF is not only associated with the survival, proliferation, and differentiation of

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https://doi.org/10.1016/j.fitote.2020.104634
Received 8 April 2020; Received in revised form 17 May 2020; Accepted 19 May 2020
Available online 23 May 2020
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melanocytes but also a crucial transcriptional regulator of tyrosinase, TRP-1, and TRP-2 expression, thereby regulating melanin production [15,16]. MITF has been shown to specifically bind to the M and *E*-box consensus motifs and to activate the tyrosinase, TRP-1, and TRP-2 promoters, up-regulating melanin synthesis [17]. In the process of melanogenesis, binding of the cAMP-elevating agent α-MSH to the melanocortin 1 receptor (MC1R) activates adenylate cyclase (AC), resulting in the elevation of intracellular cAMP levels, which stimulate the melanogenic pathway [18]. Then cAMP activates protein kinase A (PKA), which is transferred to the nucleus and phosphorylates its principal substrate CREB at Ser133 [19]. The phosphorylated CREB bond to cAMP response element (CRE) presenting in the M promoter of the MITF gene. Finally, the increase in MITF levels can lead to the up-regulation of tyrosinase, TRP-1, and TRP-2, thereby promoting melanin synthesis; de-regulation of this signaling pathway may cause pigmentation disorders.

Numerous biological antioxidants and/or tyrosinase inhibitors, such as hydroquinone, kojic acid, and arbutin have been developed to lighten the skin and/or ameliorate hyperpigmentation disorders and pathological skin discoloration [20–22]. However, these popular cutaneous depigmenting agents show some adverse side effects and problems, such as strongly irritation and cell cytotoxicity, which limit their application. Therefore, it is necessary to find safer and more effective depigmenting agents. Recent attempts have been made to develop new whitening cosmetics or skin depigmenting agents with natural ingredients from plant resources, of which polyphenols are among the strongest candidates [23].

Tea is one of the most popular non-alcoholic beverages worldwide. It is rich in natural polyphenols, and its habitual consumption has long been associated with health benefits, including antioxidant activity [24], anti-inflammatory [25], anti-cancer [26], prolonged lifespan [27], weight loss [28], liver- and neuro-protective effects [29,30]. The major active ingredients of tea plant (*Camellia sinensis* (L.) O. Ktze) are polyphenolic compounds known as catechins; tea catechins account for 60%–80% of total tea polyphenols. They primarily include EGCG, GCG, ECG, EGC, and EC, which are responsible for most of the beneficial effects of tea. EGCG is the most abundant, most active, and most well-studied catechin [31]. In recent years, the whitening and anti-melanogenic effects of tea extracts have gradually attracted the attention of researchers. Many studies have shown that tea extracts, especially their major active ingredients, exhibit excellent inhibitory effects on melanogenesis, but studies of the underlying mechanisms are not well known.

Therefore, in this study, we aimed to further analyze the depigmenting effects of tea catechins and their underlying mechanisms. We investigated whether the catechins ameliorate melanogenesis through the suppression of melanogenesis-specific enzymes other than tyrosinase and/or via upstream events in B16F10 cells. We selected GCG, EGCG, and ECG (Fig. 1) as the representative catechins and hypothesized that they functionally cause the cAMP/CREB-mediated down-regulation of MITF, which is the predominant cascade in melanin production, resulting in a reduced expression of the melanocyte enzymes tyrosinase, TRP-1, and TRP-2. Our study aids in the development of novel cosmetic ingredients, food supplements, and functional foods containing active catechins.

## 2. Materials and methods

### 2.1. Materials

B16F10 cells were purchased from Nanjing Kebai Biotechnology (China). ECG, GCG, and EGCG (purity ≥98%) were supplied by Hunan Sun full Bio-tech Co. Ltd. (China). AT was purchased from Nanjing Zelang Medical Technology Co. Ltd. (China). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Hyclone (Logan, UT, USA), fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA); 3-(4–5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sangon Biotech (Shanghai, China); 3,4-dihydroxy-*L*-phenylalanine (L-DOPA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trizol® Reagent was purchased from Life Technologies (Carlsbad, CA, USA). PrimeScriptTM RT reagent Kit with gDNA Eraser and SYBR® Premix Ex TaqTM were purchased from Takara (Kyoto, Japan). The total protein extraction radioimmunoprecipitation assay (RIPA) kit and bicinchoninic acid (BCA) kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The cAMP ELISA kit was purchased from Elabscience (Wuhan, China). Antibodies against MITF, CREB, and p-CREB were purchased from ABZOOM (Shanghai, China). Antibodies against tyrosinase, TRP-1, TRP-2, and β-actin were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The chemiluminescence (ECL) detection kit was acquired from Millipore (California, USA).

### 2.2. Cell culture

B16F10 cells were cultured in DMEM supplemented with 10% FBS at 37 °C in 5% CO2. The cells were seeded in a 96-well plate at a density of $10^4$ cells/well. When cell confluence reached 80%–90%, cells were trypsinized with 0.25% trypsin containing 0.05% ethylene diamine tetraacetic acid (EDTA) and subcultured. More than three generations logarithmic-phase cells were collected for subsequent tests, and the same-generation cells were taken for each test.

### 2.3. Morphological evaluation

B16F10 cells were seeded in a 96-well plate (104 cells/well) for 24 h and treated with different concentrations of GCG, EGCG, ECG, or AT (20 or 60 μg/mL) in DMEM for 48 h. Each replicate was included four-fold. The cells were observed with an inverted microscope (Leica Microsystems, Germany) and photographed at 100× magnification using a digital camera (Olympus, Japan) equipped with Image-Pro Plus software.

### 2.4. Cell viability assay

Cell viability was assessed by MTT assay. B16F10 mouse melanoma cells were seeded in a 96-well plate ($1 \times 10^4$ cells/well) for 24 h. Medium was replaced with 200 μL of DMEM supplemented with GCG, EGCG, ECG, or AT (10, 20, 40, 60, 80, or 100 μg/mL) and cells were incubated for 48 h. Then the medium was replaced with 90 μL of DMEM and 10 μL of 5 mg/mL MTT, and the cells were incubated at 37 °C for an additional 4 h. The medium was removed, 150 μL of DMSO was added, and the solution was incubated for 5 min with shaking. Cell viability was assessed by measuring absorbance at 570 nm with an ELISA reader (Varioskan Flash, Thermo Scientific, USA).

### 2.5. Tyrosinase activity assay

To measure the intracellular tyrosinase activity, B16F10 cells were seeded in a 96-well plate ($1 \times 10^4$ cells/well) for 24 h. The medium was replaced with DMEM containing GCG, EGCG, ECG, or AT (5, 10, 20, 40, or 60 μg/mL) and cells were incubated for 48 h. Then the medium was removed, and the cells were washed with PBS. Then 180 μL of PBS containing 1% Triton X-100 was added to every well, and the plate was shaken for 10 min. Next, 20 μL of 1 mg/mL L-DOPA was added, and the cells were incubated at 37 °C for 60 min. Tyrosinase activity was assessed by measuring absorbance at 475 nm with an ELISA reader.

### 2.6. Measurement of melanin synthesis

B16F10 cells were seeded into a 6-well plate at a density of $2 \times 10^5$

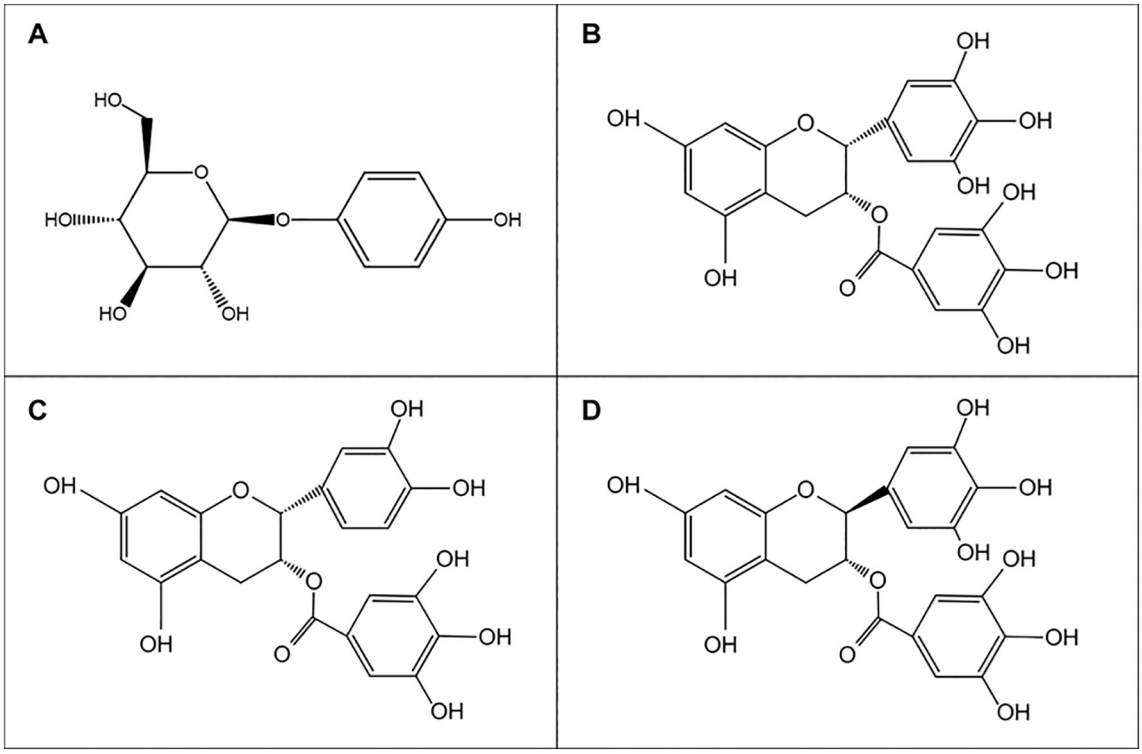


**Fig. 1.** Chemical structures of the compounds used in this study. (A) Arbutin (AT); (B) Epigallocatechin-3-gallate (EGCG); (C) Epicatechin-3-gallate (ECG); (D) Gallocatechin-3-gallate (GCG).

**Table 1**
Primers of the genes for real-time fluorescence quantitative PCR.

| Gene | Gene ID | Primer sequence |
|---|---|---|
| MITF | 17342 | F: 5'-GCTGGAGATGCAGGCTAGAG-3′<br>R: 5'-GAGAGGGCATCGTCCATCAG-3′ |
| TYR | 22173 | F: 5'-CCCAGAAGCCAATGCACCTA-3'<br>R: 5'-ATAACAGCTCCCACCAGTGC-3' |
| TRP-1 | 22178 | F: 5'-TTCATTGGCACCTGCTTTGC-3'<br>R: 5'-TCACAGCTCCAACGAAGGAC-3' |
| TRP-2 | 22064 | F: 5'-CCTACCGCCTTCGAGTCATC-3'<br>R: 5'-TCCCAGGCATAGTCAGCTCT-3' |
| β-actin | 031144 | F: 5'-CCACCATGTACCCAGGCATT-3'<br>R: 5'-AGGGTGTAAAACGCAGCTCA-3' |

cells/well. After incubation for 24 h, the cells were treated with EGCG, GCG, ECG, or AT (5, 10, 20, 40, or 60 μg/mL) and incubated at 37 °C in 5% $CO_2$ for 48 h. The cells were washed with PBS, trypsinized, and centrifugated at 1500 rpm for 10 min. The cell pellet was solubilized using 1 M NaOH containing 10% Triton X-100 at 80 °C for 1 h. The quantity of melanin was determined by measuring the absorbance at 405 nm using an ELISA reader.

### 2.7. Measurement of intracellular cAMP concentration

B16F10 cells were seeded into a 6-well plate at a density of $2 \times 10^5$ cells/well. After incubation for 24 h, the cells were treated with GCG, EGCG, or ECG (20 μg/mL) or AT (60 μg/mL) and incubated at 37 °C in 5% $CO_2$ for 48 h. Cells were washed with cold PBS and dissolved in RIPA buffer containing protease and phosphatase inhibitors on ice. Samples were centrifuged at 1000 $\times g$ for 20 min to remove impurities and cell debris, and the supernatant was used for cAMP detection The concentration of intracellular cAMP was measured using a cAMP immunoassay kit.

### 2.8. Real time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from B16F10 cells treated with GCG, EGCG, or ECG (20 μg/mL) or AT (60 μg/mL) for 48 h by Trizol reagent. After extraction, RNA was dissolved in 20 μL of DEPC-treated water, and the total RNA concentration was quantified by Nanodrop 2000 (Thermo Scientific, USA). Quality and purity of the total RNA were checked by 1% agarose gel electrophoresis; bands were visualized with an imaging system (Gene Genius, Syngene, USA). cDNA was synthesized using a cDNA synthesis kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. The cDNA template was amplified by real-time PCR using the SYBR® Premix Ex Taq™ kit, and real-time PCR detection was carried out using the fluorescence qPCR instrument Rotor Gene Q (Qiagen, Hilden, Germany). The qPCR cycling conditions were initial denaturation at 95 °C for 30 s, following 40 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 1 min, and elongation at 72 °C for 30 s. The primers for RT-qPCR (Table 1) were designed by Primer software. The β-actin gene was used as an internal control. Each experiment was performed in triplicate.

### 2.9. Western blot analysis

B16F10 cells were treated with GCG, EGCG, or ECG (20 μg/mL) or AT (60 μg/mL) for 48 h. Cells were washed with ice-cold PBS and lysed in 400 μL RIPA buffer containing 1% protease and phosphatase inhibitor for 30 min. Protein concentrations in the cell lysates were measured using a BCA protein assay kit. After mixing with 5× SDS loading buffer, equal amounts of protein (20 μg/sample) were heated for 10 min at 95 °C, separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene fluoride (PVDF) membrane (Thermo, Thermo Fisher Scientific, USA). The PVDF membrane was blocked with 5% skim milk powder in Tris-buffered saline with Tween 20 (TBST) at room temperature for 1 h. The membrane was incubated with antibodies overnight at 4 °C. The PVDF membrane was washed three times with TBST

for 10 min. Then horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, Proteintech, USA) was added, and the membrane was incubated at room temperature for 1 h. The membrane was again washed three times with TBST for 10 min, and protein bands were visualized by enhanced chemiluminescence (ECL) and quantified by Image-pro plus software (Media Cybernetics, USA).

### 2.10. Statistical analysis

All data were obtained from at least three independent experiments. Data are expressed as mean ± standard deviation (SD). Differences between the groups were evaluated by one-way analysis of variance (ANOVA) followed by homogeneity of variance test using SPSS software (version 22.0; SPSS Inc., Chicago, IL, USA). Differences were considered to be statistically significant at $P < .05$.

## 3. Results

### 3.1. Effects of catechins on cell viability and morphology

The catechins GCG, EGCG, and ECG did not significantly reduce growth of B16F10 cells at concentrations of 0–20 μg/mL, indicating that they are non-cytotoxic at low concentrations. AT was used as a positive control; AT exerted no significant inhibitory effects on cell growth at concentrations of up to 60 μg/mL (Fig. 2A).

Morphology of B16F10 cells exposed to various concentrations of catechins was examined by microscopy. Representative pictures are shown in Fig. 2B. It can be observed that the untreated control cells grew vigorously, adhered strongly, and exhibited spindle-shaped morphology and obvious cell division. At catechin concentrations of up to 20 μg/mL and AT concentrations of up to 60 μg/mL, little change in cell morphology and number was observed. However, at catechin concentrations of 60 μg/mL, the number of cells decreased significantly and some cells exhibited altered morphology. In addition, untreated control cells contained abundant black precipitates, indicative of melanogenesis. Melanin deposition was obviously decreased in cells treated with GCG, EGCG, ECG, or AT.

### 3.2. Effects of the catechins on intracellular tyrosinase activity

Tyrosinase is the rate limiting enzyme in the process of melanin synthesis, and the first step in studies of melanogenesis is generally observation of intracellular tyrosinase activity. To examine the inhibitory effects of GCG, EGCG, and ECG on intracellular tyrosinase activity, B16F10 cells were exposed to different concentrations of catechins, ranging from 0 to 60 μg/mL for 48 h. As shown in Fig. 3A, compared with the untreated control, GCG, EGCG, ECG, and AT reduced intracellular tyrosinase activity in a dose-dependent manner. GCG, EGCG, and ECG, at a concentration of 20 μg/mL, reduced tyrosinase activity by 19.05%, 22.54%, and 27.67%, respectively; the ECG-induced inhibition was statistically significant ($P < .05$). At a concentration of 40 μg/mL, all three catechins exerted statistically significant inhibitory effects on tyrosinase activity ($P < .05$). In contrast, AT, even at a concentration of 60 μg/mL, did not significantly inhibit tyrosinase activity.

These results indicate that GCG, EGCG, and ECG could inhibit melanin synthesis by inhibiting tyrosinase activity in B16F10 cells; their effects on tyrosinase activity are stronger than those of AT. ECG exhibited the strongest inhibitory effect.

### 3.3. Effects of the catechins on melanin content in B16F10 cells

To investigate the effect of catechins on melanin synthesis, B16F10 cells were treated with GCG, EGCG, and ECG concentrations ranging from 0 to 60 μg/mL, for 48 h. As shown in Fig. 3B, GCG, EGCG, and ECG reduced the intracellular melanin content in a dose-dependent manner, and their inhibitory effects were stronger than those of AT at the same concentrations. At GCG, EGCG, ECG, and AT concentrations of 20, 10, 10, and 60 μg/mL, respectively, melanin content was significantly lower than in control cells; a concentration of 20 μg/mL of either one of the three catechins was more effective than a concentration of 60 μg/mL of AT. These results indicate that GCG, EGCG, and ECG could achieve whitening by inhibiting intracellular melanin synthesis.

### 3.4. The catechins downregulates cAMP/ CREB signaling pathway

Melanin synthesis and melanogenic gene expression are regulated by various signaling pathways, among which the cAMP-mediated CREB signaling pathway is the predominant one. To elucidate the mechanisms underlying the anti-melanogenic effects of the catechins, B16F10 cells were exposed to GCG, EGCG, and ECG (20 μg/mL) and AT (60 μg/mL).

cAMP is a well-known intracellular second messenger playing a crucial role in the cAMP signaling pathway, which can affect the expression of melanogenesis-related genes. Therefore, the effects of GCG, EGCG, ECG, and AT on intracellular cAMP levels in B16F10 cells were measured. As shown in Fig. 4A, GCG, EGCG, and ECG (20 μg/mL) and AT (60 μg/mL) reduced the intracellular cAMP levels in B16F10 cells by 31.32%, 41.16%, 40.08%, and 15.42% compared to the untreated control group ($P < .01$). cAMP could stimulate the melanin synthesis pathway, which is related to the upstream activation of the phosphorylation of the CREB transcription factor, which in turn stimulates the activation of the MITF transcription factor. Thus, to identify the signaling pathways that are involved in melanin synthesis and regulated by catechins, we examined whether the catechins regulate CREB activation. As shown in Fig. 4B, the phosphorylation of CREB was significantly suppressed by the catechins and AT. Compared to the control group, catechins (GCG, EGCG, and ECG) and AT inhibited the levels of p-CREB by 24%, 30%, 44.67%, and 14% ($P < .01$), respectively, as revealed by Western blot analysis. These results suggest that the mechanisms underlying the inhibitory effects of GCG, EGCG and ECG on melanogenesis in B16F10 cells involve the inhibition of cAMP-mediated CREB signaling.

### 3.5. The catechins inhibits the expression of melanogenic genes and proteins through the down-regulation of MITF expression

To (i) determine whether the reduction of tyrosinase activity by GCG, EGCG, and ECG is related to tyrosinase expression and (ii) identify the mechanisms underlying their inhibitory effects on melanogenesis, we examined the expression levels of the melanogenesis-related genes and proteins MITF, tyrosinase, TRP-1, and TRP-2 in B16F10 cells. As shown in Fig. 5, compared to the untreated control cells, GCG, EGCG, and ECG (20 μg/mL) significantly decreased the mRNA and protein expression levels of MITF, tyrosinase, TRP-1, and TRP-2 ($P < .01$). Again, ECG exhibited the strongest inhibitory effect. AT (60 μg/mL) exhibited weaker inhibitory effects on the mRNA and protein expression levels of these enzymes; no significant difference was found in mRNA expression levels of tyrosinase between AT-treated and control cells. These results indicate that the inhibitory effect of the catechins on tyrosinase activity and melanogenesis is related to significantly decreased mRNA and protein expression levels of tyrosinase and tyrosinase-related enzymes.

It is well known that the regulation of mRNA expression of tyrosinase, TRP-1, and TRP-2, which is regulated by MITF, is crucial in melanogenesis. We hypothesized that catechins suppress melanogenesis by MITF-mediated down-regulation of the expression of tyrosinase and TRPs.

## 4. Discussion

As various factors and pathways influence melanin synthesis, many

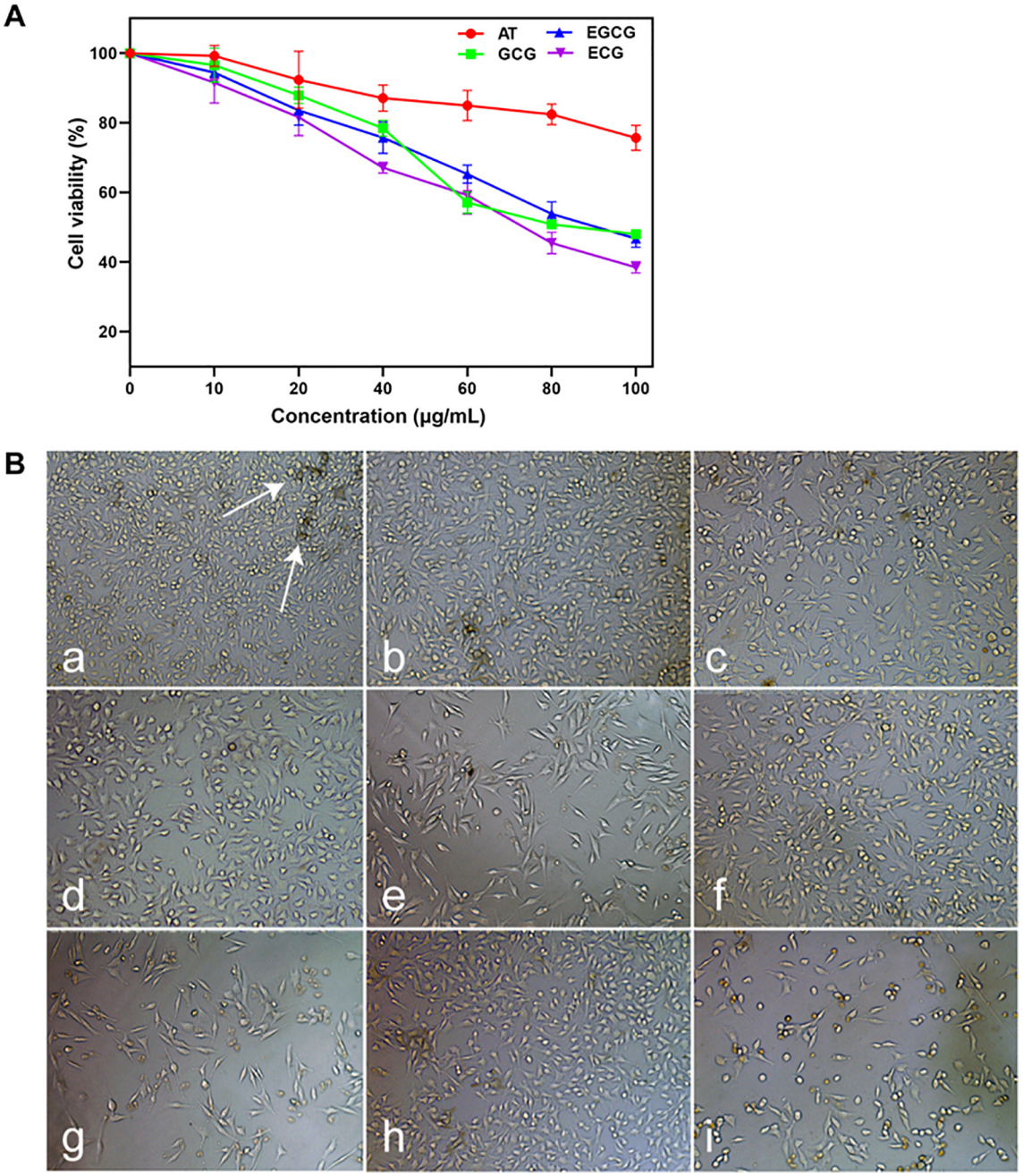


**Fig. 2.** Effects of tea catechins (GCG, EGCG, and ECG) and AT on cell viability and morphology. (A) Cell viability was measured by MTT assay in B16F10 cells incubated with catechins and AT at concentrations of 0, 10, 20, 40, 60, 80, and 100 μg/mL. (B) Morphology of B16F10 cells treated with the indicated concentrations of catechins and AT. (a) Control: cells without any treatment. (b, c) 20 and 60 μg/mL of AT. (d, e) 20 and 60 μg/mL of GCG. (f, g) 20 and 60 μg/mL of EGCG. (h, i) 20 and 60 μg/mL of ECG. The arrows show melanin secreted by melanocytes.

plant compounds with anti-melanogenic effects have been found. Tea, one of the most popular beverages worldwide, is rich in a wide range of bioactive ingredients. Tea catechins, such as EGCG, GCG, ECG, EGC and EC, are the main bioactive polyphenols in green tea and also known for their health promoting effects [32]. The beneficial health effects associated with tea catechins has been attributed in part to a reduction in the risk of developing diabetes, cardiovascular disease, cancer through multiple mechanisms including excellent antioxidant capacity by scavenging free radicals, chelating redox active transition-metal ions, inhibiting redox active [33,34].

The skin acts as a barrier protecting internal organs from the external environment, including UV radiation. UV radiation can penetrate the layers of the skin, increasing melanogenesis and the proliferation of melanocytes by directly or indirectly [35]. It is possible that tea catechins could influence a reduction in the melanogenesis and inflammation in skin, as has been highlighted in many in vitro studies using melanocyte cell lines, and in vivo studies in mice and humans. [36,37]. In addition to the reduction of melanin generation, EGCG also exhibited the positive effects on skin hydration, moisture retention, and reducing wrinkle formation. In this study, we further investigated and compared the hypopigmentation effects of the catechins GCG, EGCG, and ECG as one of the new beneficial functions and clarified their related mechanisms.

Tea catechins have been widely used as an additive in skin creams because of the physiological effects [38]. However, cytotoxicity may occur when the final catechin concentrations are excessive. To

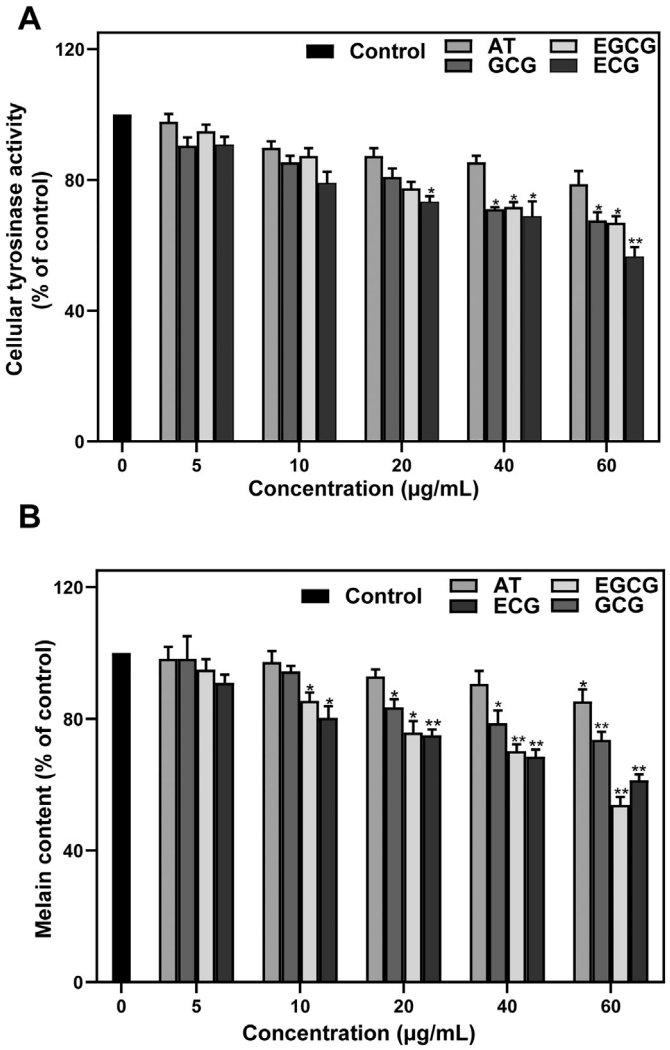


**Fig. 3.** Effects of tea catechins (GCG, EGCG, and ECG) and AT on melanin content and tyrosinase activity in B16F10 melanoma cells. Cells were treated with various concentrations for 48 h (0–60 μg/mL). (A) Tyrosinase activity. (B) Relative melanin content. $^{*}P < .05$, $^{**}P < .01$ vs. the control group.

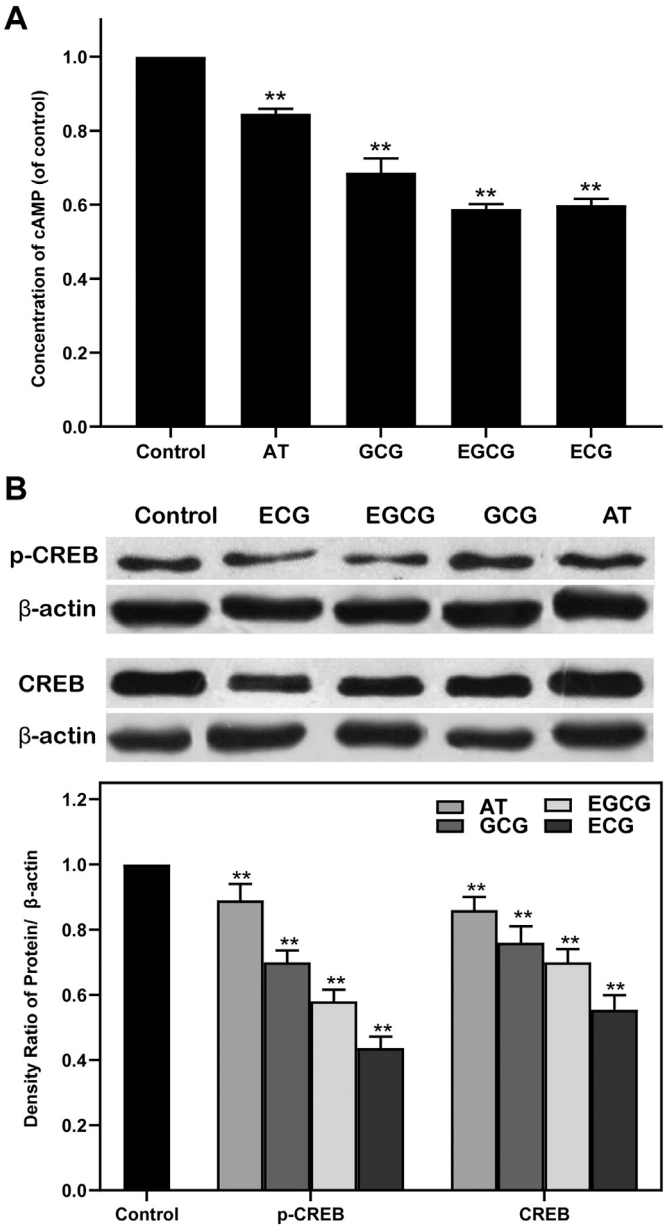


**Fig. 4.** Relative cAMP concentration and protein expression levels of CREB and p-CREB in B16F10 cells treated with the indicated compound (20 μg/mL of catechins or 60 μg/mL of AT) for 48 h. (A) The relative concentration of cAMP was measured using a cAMP Elisa kit. (B) The protein expression of p-CREB and CREB was examined by Western blot analysis. $^{*}P < .05$, $^{**}P < .01$ vs. the control group.

determine the optimum experimental concentration of tea catechins, the viability of B16F10 cells treated with different concentrations of GCG, EGCG, and ECG was evaluated by MTT assay. The results show that the catechins and AT (a well-known depigmenting agent used as a positive control) exhibit no significant cytotoxicity up to a concentration of 20 μg/mL and 60 μg/mL, respectively (Fig. 2). Moreover, melanin content and intracellular tyrosinase activity in cells treated with GCG, EGCG, ECG, and AT were reduced in a dose-dependent manner in the range of 0–60 μg/mL (Fig. 3). Therefore, concentrations of catechins (20 μg/mL) and AT (60 μg/mL) were used in subsequent experiments.

Tyrosinase, which plays a crucial role in melanin synthesis, is a copper containing glycoprotein specific to melanocytes, where it is located in special organelles called melanosomes [39]. Tea extracts have been shown to decrease tyrosinase activity in vivo and in vitro [40,41]. Tea catechins inhibited mushroom tyrosinase activity in the cell-free system, and green tea, black tea, oolong tea and white tea extracts have the inhibitory effects on tyrosinase activity in melanoma cells. Tea flavonoids, including catechins, have been reported to be powerful antioxidants and to have metal chelating properties [42]. Thus, the inhibitory effects of catechins on tyrosinase activity might be associated with their antioxidant activity and metal chelation ability. A previous study reported that the main active constituents of green tea, GCG, EGCG, and ECG, which have a galloyl group as an active site, inhibit mushroom tyrosinase activity in the cell-free system, and the inhibitory effect was in the order of GCG > ECG > EGCG [43]. The difference in inhibitory effects of the three catechins might be a result of their different molecular structures (Fig. 1).

A precise understanding of the molecular structures and the roles of chemical groups will aid in illustrating reasons behind the difference in the efficacy of different catechins. Structural features that significantly contribute to their biological action include the numbers and positions of the hydroxyl and galloyl groups on the rings, which determine their ability to interact with biological matter. Of the three tea catechins included in this study, GCG has its galloyl group in the *trans*-conformation, which may be beneficial to its inhibitory effect on tyrosinase activity. Compared with EGCG, ECG has a lower molecular weight and

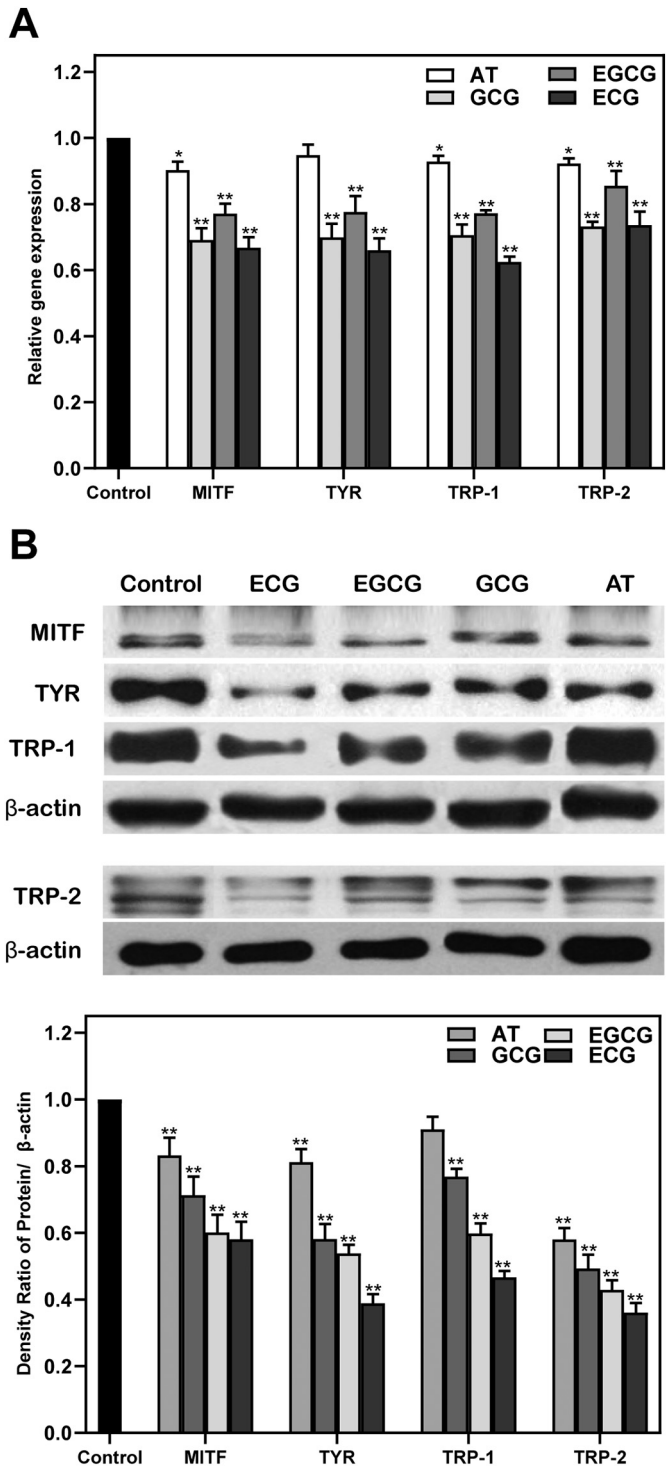


**Fig. 5.** Effects of tea catechins (GCG, EGCG, and ECG) and AT on the mRNA and protein expression levels of MITF, tyrosinase, TRP-1, and TRP-2 in B16F10 melanoma cells. Cells were treated with 20 μg/mL of GCG, EGCG, or ECG or 60 μg/mL of AT. (A) mRNA levels of MITF, tyrosinase, TRP-1, and TRP-2 were analyzed by RT-qPCR. (B) Relative protein expression levels of MITF, tyrosinase, TRP-1, and TRP-2 were analyzed by Western blot. $^{*}P < .05$, $^{**}P < .01$ vs. the control group.

may be more likely to bind to active enzyme sites when inhibiting tyrosinase activity in vitro. EGCG has one more phenolic hydroxyl group than ECG, which may cause greater spatial resistance during the interaction, weakening its inhibitory effect on tyrosinase activity in cell-free system.

However, it is interesting that in our study, the inhibitory effect on intracellular tyrosinase activity and melanin content in B16F10 cells was in the order of ECG > EGCG > GCG. The inconsistency in the order of inhibitory effect of the three catechins between the cell-free system and B16F10 cells indicates (i) Tea catechins only inhibit tyrosinase activity in cell-free system and do not involve other steps of melanin synthesis. (ii) The catechins, in particular ECG and EGCG, might inhibit melanogenesis in melanoma cells by other mechanisms, such as the suppression of melanogenesis-specific enzymes, including tyrosinase and upstream events, thereby indirectly inhibiting tyrosinase activity. Thus, tea catechins may not only inhibit the activity of tyrosinase but also inhibit the formation of tyrosinase in B16F10 cells. In addition, in this study we observed that the inhibitory effects of the three catechins on intracellular tyrosinase activity and melanin content were greater than those of AT, which is a well-known tyrosinase inhibitor that exhibits strong inhibitory effects on melanin production and is used in commercial whitening products [44], 20 μg/mL of any of the catechins caused stronger inhibition than 60 μg/mL of AT. These results indicate that the anti-melanogenic effects of the catechins are not only achieved by direct inhibition of tyrosinase activity, other mechanism are further worth investigating.

It is well known that the suppression of mRNA and protein expression of various melanogenesis-associated proteins, such as tyrosinase, TRP-1, and TRP-2, is the key mechanism by which most tyrosinase inhibitors control melanogenesis [45]. Therefore, we investigated the effects of the catechins GCG, EGCG, and ECG on the transcription and translation of these genes in melanoma cells. The catechins significantly reduced the expression levels of these genes and proteins (Fig. 4), and also of upstream proteins such as MITF. MITF is a basic helix-loop-helix leucine zipper transcription factor. MITF mutations can lead to melanocyte pigmentation disorders, loss of pigmentation, microphthalmia, and hypoplasia of the retinal pigmented epithelial cells. It plays a key role in the regulation of melanogenic enzymes, including tyrosinase, TRP-1, and TRP-2. MITF can bind to the promoters of these pigmentation enzymes and increase their expression [46]. In this study, we showed that the MITF mRNA and protein expression levels were reduced by tea catechins by RT-qPCR and Western blot analysis; the repressive effect was also in the order of ECG > EGCG > GCG. The above results suggest that the catechins could decrease melanogenesis by suppressing the expression of tyrosinase, TRP-1, and TRP-2 via down-regulation of MITF.

Although melanogenesis-associated proteins play key roles in melanogenesis, some non-enzymatic reactions can also regulate melanogenesis. A recent study showed that a decrease in MITF levels can result in the inhibition of α-MSH-induced cAMP-dependent melanogenesis in melanoma cells [47]. cAMP-mediated signaling, the predominant cascade in melanin production, is mainly affected by changes in intracellular cAMP levels [48]. When α-MSH binds to MC1R, it activates the adenylate cyclase system on the cell membrane, which converts adenosine triphosphate (ATP) into cAMP. Elevation of cAMP levels can induce the phosphorylation of CREB, which has been found to induce MITF transcription [49]. The cAMP response element (CRE) located in the promoter region of MITF binds to CREB and up-regulates the expression of the MITF gene [50]. Thus, we hypothesized that tea catechins-induced MITF downregulation is also involved in the cAMP/CREB signal transduction cascade. We found that the catechins GCG, EGCG, and ECG decrease intracellular cAMP levels and down-regulate CREB phosphorylation in melanoma cells, with the repressive order of ECG > EGCG > GCG (Fig. 5). Therefore, we further confirmed the tea catechins could inhibit melanogenesis via down-regulation of cAMP/CREB signaling, including downstream MITF expression.

## 5. Conclusion

This study reveals that the tea catechins EGCG, GCG, and ECG can inhibit melanogenesis in B16F10 melanoma cells, with the repressive

order of ECG > EGCG > GCG. The mechanisms underlying the inhibitory effects of tea catechins on melanin synthesis, in addition to the direct inhibition of tyrosinase activity, mainly involved interference with transcription and translation factors and common signaling pathways implicated in melanin synthesis. Briefly, the tea catechins downregulated MITF expression via suppression of cAMP, leading to a subsequent decrease in CREB phosphorylation and a decrease in tyrosinase, TRP-1, and TRP-2 levels, reducing melanin synthesis. Considering the low cytotoxicity of GCG, EGCG, and ECG and their superiority to AT in inhibition efficacy, we suggest that tea catechins could be applied as a potent natural depigmenting agent in the cosmetics and pharmaceutical industries.

## Authors' contributions

Jian-an Huang and Yong Lin conceived and designed the study. Xiangna Zhang carried out experiments and analyzed data. Juan Li doublechecked the data. Xiangna Zhang drafted the manuscript. Yinhua Li and Zhonghua Liu helped perform the manuscript and provided assistance for literature search manuscript editing. All the authors have read and approved the final content of the manuscript.

## Declaration of Competing Interests

The authors declare that there are no conflicts of interest.

## Acknowledgments

This work was supported by the National Major R & D Project in China (grant number: 2017YFD0400803) and the China Tea Research System Project (grant number: CARS19-09B) and Scientific Research Fund of Hunan Provincial Education Department (19A223).

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

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