# Inhibitory Activity of Soy Cell Culture Extract on Tyrosinase Activity and Melanin Formation in  -MSH Induced B16-F10 Melanoma Cells

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# Abstract

**Background** Hyperpigmentation particularly in the cosmetic industry is one of the most important issue that causes excess production and accumulation of melanin. Nowadays, some materials such as kojic acid and its derivatives and arbutin as a skin whitening are used to overcome hyperpigmentation. Since they have side effects such as skin irritation and contact dermatitis, the new surveys focus on agents which are natural and have no side effect.

**Methods and Results** In the present study, we investigated that effect of soy cell culture extract on α-MSH induced melanogenesis in B16-F10 melanoma cells.

We found that the soy cell culture extract supressed melanin sythesis and showed anti tyrosinase activity on B16-F10 melanoma cells in a dose dependent manner without cytotoxicity. In addition, we determined that soy cell culture extract suppressed the expression of TYR, TRP1, TRP2 and MITF at both the gene expression and protein level.

**Conclusions** In conclusion, according to the current results, soy cell culture extract has high potential for using as an ingredient in skin whitening products.


# 1. Introduction

The epidermal–melanin unit, a functional unit that produces and distributes melanin, is composed of one

melanocyte and approximately 36 neighboring keratinocytes [1]. Melanocytes are responsible for melanin production and distribution via a process called melanogenesis [2]. Enzymatically, tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP-2, also called dopachrome tautomerase) are three key regulators of the melanogenesis [3]. The tyrosinase family genes TYR, TRP-1, TRP-2 responsible for pigmentation are transcriptionally regulated by microphthalmia-associated transcription factor (MITF) [4].

Melanin, the major pigment of skin and hair color in mammals, protects human skin against a harmful

ultraviolet radiation (UVR) and stress from the different source of environmental pollutants, toxic drugs, and chemicals [3, 5]. Although melanin is critical in protecting the skin, abnormal production of melanin can lead to pigmentary disorders such as hypopigmentation and hyperpigmentation. While hypopigmentation is observed in vitiligo and albinism, hyperpigmentation causes dermatological problems such as melasma, reckles, post inflammatory hyperpigmentation and age spot [6].

Recently, researchers have developed tyrosinase inhibitors and biological reductants such as kojic acid,

arbutin, hydroquinone and ascorbic acid to treat hyperpigmentation and pigmentation diseases. However, the use of skin whitening agents that can suppress tyrosinase is limited due to low stability in the presence of oxygen and water, and the side effects such as cytotoxicity, skin irritation and dermatitis [7].

For these reasons, there is a need for alternative new skin whitening agents that contain natural compounds that are more effective and have fewer side effects [5].

Nowadays, the plant extracts are becoming the most popular active ingredients of cosmetics due to the

ever-increasing demand for natural compounds which in addition to esthetic looks can provide additional health benefits [8].

On the other hand, plants of cosmetic interest have been limited for use due to slow growth, seasonal

harvest, variation of active concentration from plant to plant and harvest to harvest and existence of toxic metabolites. Plant cell culture techniques help to overcome these important problems related to the plant use [9]. It is possible to produce standardized and season-independent production under controlled conditions using plant cell culture technology. It is ecofriendly as has a low impact on the ecosystem, requires less water use, reduces carbon footprint and does not require pesticides and herbicides [10]. Because of all these advantages, in the current study we used plant cell culture extract instead of conventional agricultural extract to determine the potential of soy as a skin whitening agent. Plant cell culture technology also enables phytochemicals such as phenolic compounds found in plants to be obtained at high yields [9]. Along with many biological activities of phenolics, anti-tyrosinase and melanin-inhibition activities have also been reported [11]. Phenolic compounds are widely distributed in nature and are the largest groups in tyrosinase inhibitors and the best-studied polyphenols are flavonoids [12]. The effective anti-tyrosinase and melanin synthesis inhibition activities of many isoflavonoid and other phenolic compounds found in soy plant have been demonstrated in various studies [13–18]. Despite these reports with phenolic compounds found in soy, the effects of extract of soy plant or soy cell suspension on anti-tyrosinase and melanogenesis have not yet been revealed. Therefore, it was aimed to determine the effects of soy cell culture extracts on the process of melanogenesis.

# 2. Material And Methods

### Reagents

MS medium (MSP09), 2,4 D (D001 and gelrite (G024) were purchased from Caisson Lab (USA). Tween 20 (P1379), gallic acid (G7384) kojik acid (K3125), α-MSH (M4135) and L- DOPA (D9628) were obtained from Sigma Aldrich (St Louis, MO, USA).

MTT (A2231) and DMSO (A3672) were purchased from Applichem (Darmstadt, Germany). DMEM (41966-029), IMDM (21980-032) and FBS (10270-106) obtained from Gibco Life Technologies (Carslbad, CA, USA).

RIPA buffer and RNA isolation kit and cDNA synthesis kit were purchased from respectively Thermo Fisher Scientific (Waltham, MA, USA), Invitrogen (Carlsbad, CA, USA) and Applied Biosystems (Forster City, CA, USA). BCA assay kit and Blocking kit (iBlot Transfer Stacks, nitrocellulose) and chromogenic detection kit (Western Breeze Chromogenic kit) were obtained from Thermo Fisher Scientific (Waltham,

MA, USA). ACTB (Sc-47778), TYR (Sc-20035), TRP1 (Sc-166857), TRP2 (74439) and MITF (Sc-56725) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

## Plant Cell Culture

*Glycine max* (soybean) seeds were surface-sterilized for 20 min in a 20% solution of commercial bleach containing 1-2 drop Tween 20, followed by 4 times rinse with sterile distilled water and incubated onto solid MS medium in the dark at 25 ºC for the germination. After 7 days, the hypocotyl explants were cut from germinated seedling and place onto solid MS medium supplemented with 1mg/L 2,4 D and 30 g/L sucrose. Friable calluses were used to establish cell suspension culture. Cell suspension culture was initiated by agitation 4 g of callus in 250 ml flasks containing 50 ml of liquid MS medium supplemented with 1 mg/L 2,4 D and 30 g/L sucrose at a shaking speed of 110 rpm. Cells were maintained by subculturing to fresh medium once every 14 days.

The cells were filtered and rinsed with distilled water. The fresh cells were lyophilizated. For the extraction 20 gram of the lyophilized cells were incubated with 200 ml 70% ethanol placed in the rotary shaker for 24 hours at room temperature. The extract were centrifuged at 10000 rpm for 10 minutes, and supernatant was filtered through PTFE syringe filter (0.22 µM). The fitrate was evaporated at 40 ºC under reduced pressure.

## Determination of total phenolic content

The concentrations of phenolic compounds in the extract of soy cell culture were measured according to Folin Ciocalteu reagent method as decribed by Ainsworth and Gillespie [19]. Briefly, 0,1 ml of soy cell culture extract (1 mg/ml) and 0,2 ml of Folin-Ciocalteu's reagent mixed together. Then, 0,8 ml of 700 mM $Na_2CO_3$ was added and the reaction mixtures were incubated for 2 h at the room temperature in the darkness. The absorbance was measured at 765 nm with a spectrometer. A dose response linear regression was generated by using the gallic acid standart absorbance (100-500 µM) and the levels in the extract were expressed as gallic acid equivalents (µM of GAEs/g of extract).

## Cell viability assay

B16-F10, mouse melanoma cells were cultured in DMEM supplemented with 10% (v/v) of FBS at 37 ºC in a humidified atmosphere containing 5% $CO_2$. Cell viability was determinated using the MTT assay to the method described by Mosmann [20]. B16F10 cells were plated into 24 well plate at a density of 0,7 $x10^5$ cells per well and incubated 24 hours. After the cells were treated with different concentrations of soy cell culture extract (SCE) for 48 h, then treated with MTT solutions (5 mg/ml) for 3 h after which the precipitated formazan was dissolved by DMSO. The optical density was measured at 570 nm with a microplate spectrophotometer.

## Melanin contents assay

Cellular melanin content was determinated using a minor modification of a method described by Jin et al. [21]. Briefly, B16F10 cells were seeded at a density $1x10^5$ cells/well on the six well plate and, after 24 h of incubation the cells were treated with 100 nm α-MSH for 24 hours except control group. After treated with α-MSH, the medium was removed and the cells were treated with of SCE and kojic acid for 48 h. After incubation, pellets were dissolved in 1 N NaOH containing 10% DMSO for 1 hour at 80 ºC. The optical density of homojenizates were read at 475 nm using a microplate reader.

### Cellular tyrosinase activity assay

The effect of SCE on cellular tyrosinase activity assay α-MSH induced tyrosinase activity in B16F10 cells were investigated following a slightly modified previous method [5]. Briefly, B16F10 cells were seeded at a density $1x10^5$ cells/ well on the six well plate and, after 24 h of incubation the cells were treated with 100 nm α-MSH for 24 hours except control group. After treated with α-MSH, the medium was removed and the cells were treated with of SCE and kojic acid for 48 h. After then the cells washed with PBS and lysed with including 1% Triton X-100 and 100 µM PMSF in PBS and centrifuged at 17500 g for 10 min at 4 ºC. Each 90 µl supernatant and 10 µl L-Dopa (2 mg/ml) was combined in the well of 96-well plate and incubated for 30 min at 37 ºC.

The oxidation of L-DOPA to dopachrome was measured at 475 nm with the absorbance reader.

### Total RNA isolation and RT-qPCR analysis

B16F10 melanoma cells were cultured at a density of $2,5x10^5$ in $25cm^2$ flasks and, after 24 h of incubation the cells were treated with 100 nm α-MSH for 24 hours except control group. After treated with α-MSH, the medium was removed and the cells were treated with of SCE for 48 h. Total RNA was isolated by using Pure RNA mini kit (Life Technologies) and total RNA was converted to cDNA using a cDNA kit according to manufacturer's protocol (High Capacity cDNA Revers Transcription Kit, Applied Biosystems). 100 ng of cellular RNA was reverse-transcribed into cDNA. RT-qPCR analysis were performed with TaqMan probes (ACTB; Assay ID: 316568, TYR; Assay ID: 316568, TRP1; Assay ID: 316563, TYRP2; Assay ID: 317497 and MITF; Assay ID: 315218, RealTime Ready Custom Single Assays, Roche) using a RT-qPCR system (LightCycler 480, Roche). Data were analyzed using the $2^{[-\triangle\triangle ct]}$ method [22] and presented as the means ±SD normalized to ACTB.

### Western blot analysis

B16F10 cells were treated with SCE and kojic acid for 48 h after treatment with 100 nM α-MSH for 24 h. The proteins were isolated with RIPA buffer and amounts of total protein was determinated with BCA assay kit (Thermo Fisher Scientific Inc., USA). Then the proteins were separated by SDS page electrophoresis using %4-12 Bis-Tris gel. Following electrotransfer to nitrocellulose membranes with dry blotting (iBlot2 Dry Blotting System, Thermo Fisher Scientific) the membranes were blocked according to manufacturer's protocol (Western Breeze Chromogenic kit, Thermo Scientific). Then the membranes incubated overnight with primary antibodies (ACTB; 1:1000, TYR ;1:50, TYRP1; 1:50, TYRP2; 1:100; MITF;

1:50) at 4 ºC and secondary antibody. The membrans were incubated with chromogenic substrate until the bands developed on the memrane.

### Statistical analysis

Graphpad Prism version 8.3 was used for the statistical analysis of *in vitro* cytotoxicity, melanin content, cellular tyrosinase activity and RT-qPCR experiments. Data were expressed as the mean ± SD values of last three independent experiments. Statistical significance was analyzed using One-Way ANOVA and Tukey's test and a P value <0,05 was considered to indicate statistical significance.

## 3. Results

### Callus and suspension culture

Friable callus formation from hypocotil explants of soy plants was observed by 2 weeks after the explants were placed in the callus induction media. For establishment of cell suspension culture, two-months-old callus was suspended in liquid media. After 10 days, aggregates were removed from suspension by sieve filtration. Soy cell suspensions were subcultured at 2 weeks periods and PCV (Packed Cell Volume) value of soy suspension culture was obtained as %50 (Fig. 1).

### Total Phenolic Content

The total phenolic content was determined from a standard curve using gallic acid (100-500 µM) as a standard and expressed as µM of gallic acid equivalents per grams of SCE weight (µM GAE/g)

Total phenolic content in 70% ethanol extract of soy cell culture was found 275 ± 13,3 µM GAE/g extract (Fig. 2). The levels in the SCE were expressed as gallic acid equivalents.

### Effect of SCE on cell viability

To investigate the effect of SCE on melanogenesis, it was first analyzed whether SCE was cytotoxic for B16F10 cells. Cell viability experiments were performed using the MTT assay. B16-F10 cells were treated with concentrations of SCE (0,1, 0,3, 0,5 and 1 mg/ml) for 48 h. As shown in Fig. 3a, there were no significant effects on the cell viability at all concentrations relative to the untreated control group. Therefore, the highest concentration, 1mg/ml of SCE was used for further studies.

### Effect of SCE on melanin contents

To determine the effects of SCE on melanin synthesis, melanin content was analyzed in B16F10 melanoma cells treated the concentrations of SCE for 48 h after incubated with 100 nm α-MSH for 24 h. Kojic acid was used as a positive control. As shown in Fig. 3b, SCE treatment significantly decreased the melanin content of cells in a dose-dependent manner compared to only α-MSH treated group. SCE at 0,3 mg/ml and at 1 mg/ml decreased melanin content by 20% and by 34% compared to only α-MSH treated group respectively. Kojic acid at 200 µM and at 400 µM reduced melanin content by 22% and by 29%

compared with α-MSH treated control group respectively. The concentration of SCE and kojic acid showed similar effects on B16F10 melanoma cells compared to control group in terms of the melanin content.

### Effect of SCE on the cellular tyrosinase activity

To examine the effect of SCE on tyrosinase activity, intracellular tyrosinase activity was determinated in B16-F10 melanoma cells treated with the concentrations of SCE for 48 h, after incubated with 100 nm α-MSH for 24 h. Kojic acid was used as a positive control. As shown in Fig. 3c, SCE treatment significantly decreased tyrosinase activity of cells in a dose-dependent manner compared to the only-α-MSH treated group. SCE at 0,3 mg/ml and 1 mg/ml reduced tyrosinase activity by 19% and by 24% compared to α-MSH control group respectively. Kojic acid at 200 μM and at 400 μM inhibited tyrosinase activity by 18% and by 24% respectively compared with only-α-MSH treated group. The concentration of SCE and kojic acid showed similar inhibitor activity on B16-F10 melanoma cells compared to control group in terms of tyrosinase activity.

### Effect of SCE on expression levels of melanogenic genes and proteins

To clarify whether SCE affects melanin synthesis and tyrosinase activity transcriptionally we analyzed expression levels of melanogenesis related genes including TYR, TRP1, TRP2 and MITF with RT-qPCR. B16-F10 cells were treated with concentrations of SCE for 48 h after treatment with 100 nm α-MSH for 24 h. As shown in Fig. 4, SCE at 0,5 and 1 mg/ml concentrations significantly and dose-dependently downregulated expression levels of TYR, TYRP1, TYRP2 and MITF genes compare to only-α-MSH treated group.

Expression of the TYR gene increased 3.3-fold in α-MSH-treated cells compared to the control group. On the other hand, expression levels of TYR gene decreased by 1.4-fold at the 0,5 mg/ml and by 1.5-fold at 1 mg/ml concentrations of SCE compared to cells treated with only α-MSH. While the expression level of the TRP1 gene didn’t change with α-MSH stimulation comparing to the control group, the expression level of the TRP1 gene reduced by 1.1-fold at the 0,5 mg/ml of SCE and 1.5-fold at the 1 mg/ml of SCE. It was observed that α-MSH stimulation increased the expression of the TRP2 gene by 1.5-fold compared to the control group. Furthermore, 0,5 mg/ml and 1 mg/ml concentrations of SCE decreased the expression level of TRP2 gene by 1.3 and 2-fold, respectively. The expression level of the MITF gene was increased by 2.8-fold in α-MSH stimulated cells compare to the control cells, SCE extract at 0,5 mg/ ml and 1 mg/ml downregulated the expression of the MITF gene by 1.5-fold and by 2-fold, respectively. SCE significantly inhibited the expression of MITF, which plays a critical role in TYR, TRP1 and TRP2 gene expression and melanogenesis.

In addition to RT-qPCR analysis, expressions of these melanogenic enzymes and transcription factor were evaluated by applying western blot analysis. SCE treatment reduced expression of MITF, TYR, TRP1 and TRP2 proteins in a dose-dependent manner (Fig. 5). It was observed that expressions of the proteins decreased significantly at 1mg/ml doses of SCE. While SCE inhibited expression of melanogenic proteins,

kojic acid did not show any inhibition effects on these proteins expressions. These results suggested that SCE inhibited melanin synthesis via MITF mediated pathway.

# 4. Discussion

Melanin is the main component determining the color of skin and its major role is to protect the skin from damaging effects of ultraviolet radiation [23]. Although appropriate melanogenesis provides effective protection against UV, excessive melanin production and accumulation lead to various dermatological disorder such as freckle, melasma, spot and other hyperpigmentation syndromes [7, 24]. Since hyperpigmentation is an important issue in the cosmetics industry, there is a great demand for melanogenesis inhibitors [2].

The most commonly used agents against hyperpigmentation include hydroquinone, kojic acid, arbutin, ascorbic acid and retinoic acid, but they have recently been reported to include several side effects [25, 26]. Therefore, there is a need to develop new tyrosinase inhibitors from different sources with high efficacy and less adverse side effects [12]. In addition, consumers want to have effective, safe, natural and sustainable cosmetic products whose production does not adversely affect the environment [10]. For these reasons, in the current study, we aimed to determine the potential use of soy cell culture extract which is a natural isoflavonoid source as an inhibitor of hyperpigmentation accumulation.

In the current study, the total phenolic content of soy cell extract was determined as 275 ± 13.3 μM GAE/g extract. In a similar research, Sansanelli et al. [27] investigated the effect of cell inoculation rate of soy suspensions on the total amount of phenolic compounds. They reported that the highest total phenolic amount was obtained as 36.2 mg GA equivalent /L in 40 grams/L inoculated suspension. In their reseach, the total phenolic amount in fresh cell was announced, the total phenolic amount in the dry cell or extract that obtained from dry cells did not given. In several studies the total phenolic amonts were examined in extracts obtained from different tissues of soy plant, other than in the soy cell culture as we did in the current study [28, 29]. However, in our study, total phenolic compound of soy cell extract was obtained in an amount (46.8 mg GAE / gram extract=275 ± 13.3 μM GA/g extract) that is close to the total phenolic amount (42.2-50.4 mg catechin/gram extract of whole soy plant) obtained in the studies of Peiretti et al. [30].

Cytotoxicity of SCE was analyzed in B16-F10 melanoma cells before evaluating the effect of its on melanogenesis. It was determined that the SCE did not show any cytotoxic effect on B16-F10 melanoma cells up to 1 mg/ml. As a first step to determining the effect of SCE on α-MSH induced melanogenesis, we investigated whether SCE could inhibit melanin synthesis after stimulation of cells with α-MSH. SCE significantly inhibited melanin synthesis and tyrosinase activity in a dose dependent manner without cytotoxicity. Then we performed mRNA analysis of melanogenic genes to determine whether SCE's activity to suppress melanin synthesis is transcriptional. For that purpose, we evaluated the effect of SCE on the expression levels of TYR, TRP1 and TRP2, which are essential genes for melanogenesis and MITF that their regulator. SCE significantly downregulated expression levels of TYR, TRP1, TRP1 and MITF in a

dose dependent manner. We also analyzed the effect of SCE on the expression of TYR, TRP1, TRP2 and MITF proteins by western blotting. SCE decreased the expression of proteins depending on the dose dependent manner. In particular, we observed that SCE at 1mg/ml concentration significantly reduced the expression of TRP1 and TRP2 proteins.

Kojic acid was used as negative control in protein studies. Kojic acid, used as skin-whitening agent is the most studied inhibitor of tyrosinase. It is well known that kojic acid chelates the tyrosinase enzyme and has a competitive inhibitory effect [12, 31]. In our knowledge, there is no data showing that kojic acid transcriptionally suppresses melanogenesis. We observed that in contrast to SCE, kojic acid concentrations did not decrease the expression of any protein involved in melanogenesis.

# 5. Conclusions

In conclusion, the present findings have shown that SCE significantly inhibits α-MSH induced melanogenesis in B16F10 melanoma cells by supressing expression of TYR, TRP1 and TRP2 proteins via inactivation of MITF transcription factor. The results indicate that soy cell culture extract might be useful therapeutic agent for in the treatment of hyperpigmentation and provide effective ingredient in skin whitening cosmetic products.

# Declarations

### Compliance with Ethical Standards

**Disclosure of potential conflicts of interest** The authors declare that they have no competing interests**.**

**Research involving human participants and/or animals** Not applicable.

**Informed consent** Not applicable.

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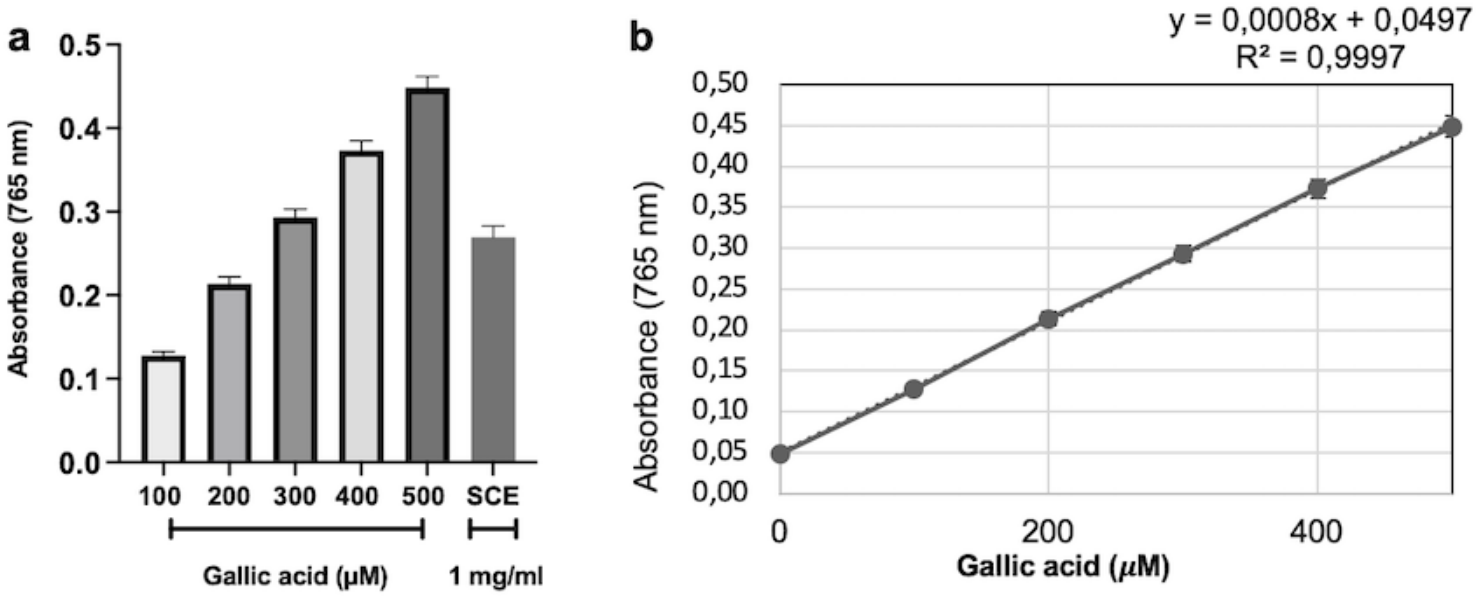
# Figures

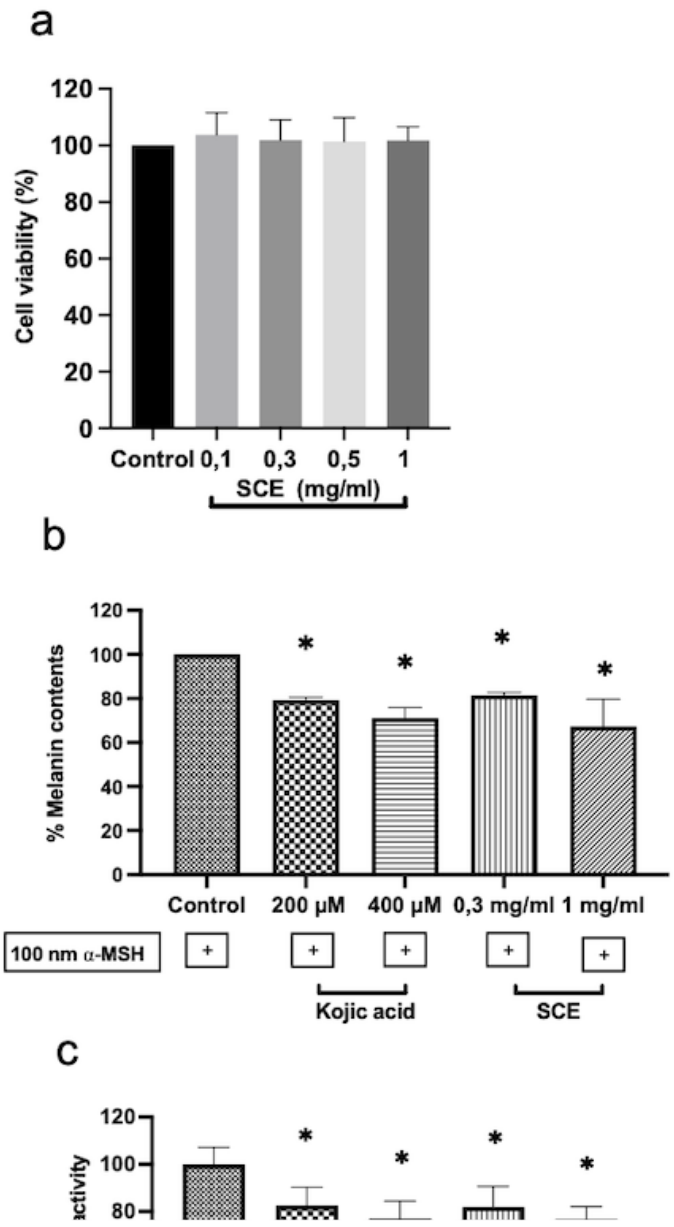

**Figure 1**

**a** *In vitro Glycine max* plant. **b** 14 days-old callus culture. **c** 2-mounts-old soy callus culture. **D** Soy suspension culture in the flasks



## Figure 2

**a** Standart curve of gallic acid at 765 nm. **b** Absorbance of SCE at 765 nm. Total phenolic content of SCE at 1 mg/ml was determined by Folin-Ciocalteu method. Each bar represents the mean ±SD (n:3)

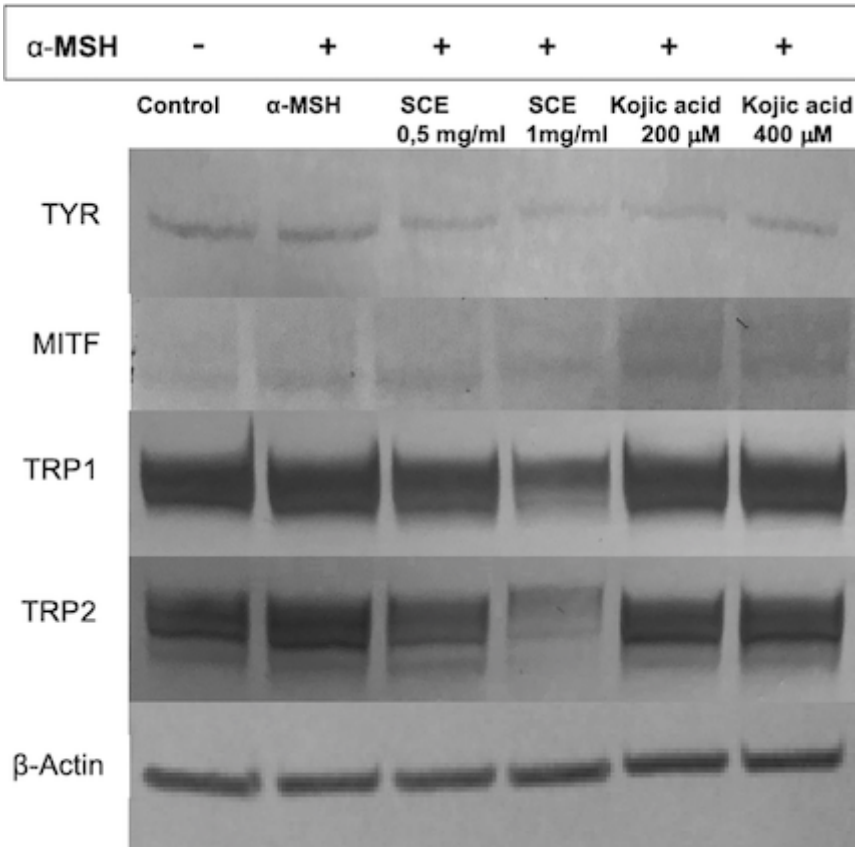



## Figure 3

**a** Effect of SCE on cell viability of B16F10 cells. The cells were incubated with SCE for 48 h. Each bar represents the mean ±SD (n:3). **b** Effects of SCE and kojic acid on melanin content in B16F10 melanoma cells. **c** Effects of SCE and kojic acid on tyrosinase activity in B16-F10 melanoma cells. B16-F10 melanoma cells in all group were treated with 100 nm α-MSH for 24h. After that the cells, except for control group were incubated with SCE and kojic acid for 48 h. *: indicates a significant difference compared to control group (p<0,01)

## Figure 4

Effect of SCE on expression level of melanogenic genes, **a** TYR, **b** TRP1 **c** TRP2, **d** MITF in B16F10 cells. The cells were treated with 100 nm α-MSH for 24 h. After that the cells except for control group were incubated with SCE for 48 h. Data were analyzed using the $2[-^{\triangle\triangle ct}]$ method and presented as the means ±SD normalized to β-Actin. #: indicates a significant difference compared to the control (p<0,01), *: indicates a significant difference compared to α -MSH treated-only group (p<0,01)




## Figure 5

Effects of SCE and kojic acid on protein expression of TYR, TRP1, TRP2 and MITF in B16-F10 cells. The cells were treated with 100 nm α-MSH for 24 h. After that the cells except for control group were incubated with SCE and kojic acid for 48 h. Expression of β-Actin was used as an internal control.