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Melanogenesis inhibition effect of ethanolic *Andrographis paniculata* leaf extract via suppression of tyrosinase and MITF expression

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ABSTRACT

Andrographis paniculata is a well-known and highly used potential herbal plant for worldwide medicinal use. Plant studies reported that the leaf extract of *A. paniculata*, which has high antioxidant capacity, has antifungal, antimicrobial, antiprotozoal, antidiabetic, liver enzyme modulation, insecticidal, and toxicity properties. Nevertheless, no report regarding its antimelanogenic activity is available. Hence, this study was carried out to investigate *A. paniculata* leaf extract potential of the antimelanogenic property. The data shown from this study proved that the *A. paniculata* leaf extract is responsible for melanin content reduction and decreases the protein expression of tyrosinase (TYR) and microphthalmia-associated transcription factor in B16F1 melanoma cells. This study found that the *A. paniculata* leaf extract acted powerfully in the synthesis of tyrosinase-related protein-2 inhibition. TYR is a well-known essential enzyme in melanogenesis activity. These results represent that the *A. paniculata* leaf extract strongly decreases the expression level of the enzyme important for melanin synthesis. Therefore, the *A. paniculata* extract has a high capability of becoming a lightening agent and hyperpigmentation treatment in the pharmaceutical and cosmetic market.

INTRODUCTION

Andrographis paniculata (Burm. f) Nees (Acanthaceae) is traditionally used for the treatment of skin diseases. This medicinal plant contains major bioactive components such as diterpenoids, flavonoids, and polyphenols (Xu *et al*., 2010). Andrographolide is the major diterpenoid in *A. paniculata*, making up about 0.5%–6% of the dried leaf extract (Chao *et al*., 2021; Loureiro Damasceno *et al*., 2021. Deoxyandrographolide, neoandrographolide, isoandrographolide, and 14-deoxy-11,12-didehydroandrographolide are the other main diterpenoids in this plant (Liu *et al*., 2020). Studies discovered that diterpene andrographolides induced significant stimulation of antibody production and delayed the hypersensitivity response toward sheep red blood cells immunized mice (Singh, 2016). The study suggested that other compounds besides andrographolides existing in the crude extracts might as well contribute to immunostimulation. The extract also shows potent cell differentiation activity in mouse myeloid leukemia cells (Bello *et al*., 2018; Pfisterer *et al*., 2010). Andrographolide, the main compound in the *A. paniculata* leaf extract, is reported to inhibit the inflammatory mediator, thus providing anti-inflammatory activity through its ability to suppress vasodilation (Rahmi *et al*., 2022). Andrographolide is helpful in skin problems, such as dermatitis, skin irritation, skin redness, dry skin, and rashes (Hossain *et al*., 2021). The antimicrobial property of andrographolide is beneficial for oily skin treatment. Other than inhibiting the production of nitric

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oxide, andrographolide is able to reduce endothelin levels (Lin *et al*., 2017; Shu *et al*., 2020). Endothelin is involved in a number of processes, including the production of melanin. These findings suggested that andrographolide from *A. paniculata* might have the ability to modulate the melanogenesis mechanism by elevating or preventing the tyrosinase (TYR) oxidation process. In addition, no study regarding the *A. paniculata* leaf extract particularly focuses on melanogenesis activity. Therefore, this study believed that the *A. paniculata* leaf extract might have the potential to be used as an ingredient in cosmeceutical products to treat skin problems. The *A. paniculata* leaf extract has been used in various cosmetic products as an ingredient. Nevertheless, there are no scientific reports on the whitening or lightening effect of the *A. paniculata* leaf extract. Studies reported melanin production can be activated by reactive oxygen species (ROS) (Denat *et al*., 2014; Kamiński *et al*., 2022). Based on this finding, this study was used to evaluate the potential of the antimelanogenic property of the *A. paniculata* leaf extract. The function of epidermal melanocytes of the skin is melanin synthesis. The main key to melanin production is protecting the skin from UVA and UVB radiation. However, exposure to continuous UV radiation results in constant melanin production. This accumulation of melanin will lead to hyperpigmentation skin problems (Lee, 2021).

α-Melanocyte-stimulating hormone (α-MSH) is a well-known melanogenesis inducer (Singh and Mukhopadhyay, 2014). The expression of microphthalmia-associated transcription factor (MITF) will be enhanced and induced by α-MSH binding to melanocortin 1 receptor (Oh *et al*., 2022). Consequently, cyclic AMP will act and elevate the expression of TRY melanogenesis-related enzymes such as tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2). Hyperpigmentation problems relate to nonesthetically pleasing issues, such as freckles, melasma, and age spots (dos santos Videira *et al*., 2013). The essential enzymes in melanin regulation are TYR and TRP-1 and TRP-2 (Xue *et al*., 2018). The main role of TYR in melanin synthesis is being a copper-containing glycoprotein (Pillaiyar *et al*., 2017).

Jeon *et al*. (2018) reported that TYR incorporates in the catalysis of four different reactions. Firstly, tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine (DOPA). Secondly, DOPA is oxidized into dopaquinone. Thirdly, dopaquinone oxidizes into dopachrome. Finally, dopachrome develops as either indole 5,6-quinone-2-carboxylic acid dihydroindolizine carboxylic acid (DHICA) or dihydroindolizine. Meanwhile, TRP-1 and TRP-2 play important roles in melanogenesis synthesis.

The catalysis of DHICA oxidation is done by TRP-1 and simultaneously TRP-2 responsible on catalyzation of dopachrome converted to DHICA (Sato *et al*., 2016). A specific transcription factor, MITF, is responsible for the regulation of melanogenesis enzymes (Kawakami and Fisher, 2017). MITF is important in the activation of diverse signaling pathways and is controlled by mitogen-activated protein kinases (MAPKs) (Cargnello and Roux, 2011; Munshi and Ramesh, 2013). MAPKs are constituted of protein serine/threonine kinases. They mostly participate in the signal transduction pathway, promoting cellular growth activities, such as differentiation and proliferation (Hu Frisk *et al*., 2018; Ngeow *et al*., 2018). The main three known characterized subfamilies contained in the MAPK superfamily are extracellular signal-regulated protein kinases (ESRPKs), phosphorylation of p38 MAPK, and c-Jun N-terminal kinases (Olea-Flores *et al*., 2019). The essential role, especially in melanogenesis regulation, is played by MAPKs (Sun *et al*., 2020).

Furthermore, ESRPK activation is done at the 73rd serine residue by c-Kit simulation that phosphorylates MITF. MITF ubiquitination and degradation are accompanied by MITF phosphorylation at the 73rd serine (Paruchuru *et al*., 2022).

Moreover, the activation of p38 MAPK can escalate the synthesis of melanin (Sun *et al*., 2020). Melanogenesis inhibition is correlated with TYR activity downregulation by ESRPK signaling activation (Song *et al*., 2015). There are some well-known melanogenesis inhibition agents used, such as arbutin, linoleic acid, and kojic acid.

Arbutin is a glycosylated hydroquinone known as glycoside (Pop *et al*., 2009). It is commonly based on a bearberry plant extract. It has been widely used for the treatment of pigmentation problems, such as melasma and spots. Arbutin and kojic acid are greatly used as cosmetic constituents based on TYR inhibition activity (Pillaiyar *et al*., 2017).

Nonetheless, an adverse reaction is the main concern in using these agents. Those with overly sensitive skin may suffer from redness and irritation. Kojic acid was reported to be able to cause irritation to the skin and cause serious skin problems, such as dermatitis and skin cancer (Chang *et al*., 2010). Exogenous ochronosis and perdurable depigmentation were reported after long-term use of arbutin (Bhattar *et al*., 2015; Sunkara *et al*., 2020).

Consequently, for the best interest of the public use of these agents, a new future ingredient should be found and extracted from natural-based ingredients or plants, which importantly will cause no harm or side effects on the skin.

The current study focused on the investigation of finding a potential melanogenesis inhibitor compound from this medicinal plant. In our study, we discovered the antimelanogenesis effect of the *A. paniculata* leaf extract and andrographolide on α-MSH-induced melanogenesis in B16F1 mouse melanoma cells. Molecular mechanisms involved in this process were also discovered in this study. Many previous studies on the *A. paniculata* leaf extract have mainly determined its antimicrobial, anti-inflammatory, and antioxidant activities.

Nevertheless, in this current research, we conducted a study on melanogenesis using the *A. paniculata* leaf extract. The results determined from this study will be applied as primary research evidence and can be used in the development of cosmetic ingredients.

MATERIALS AND METHODS

Materials and reagents

The rabbit polyclonal MITF antibody, rabbit polyclonal TYR antibody, and rabbit polyclonal TRP-2 antibody were purchased from Cusabio (Houston, TX, USA). The Folin–Ciocalteu reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma,88,415), 100% Triton™ X-100 (Sigma, T8,787), 3,4-dihydroxy-*L*-phenylalanine (L-DOPA) (Sigma, D9,628), kojic acid, α-MSH, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (USA). Dulbecco's modified eagle medium

(DMEM), penicillin–streptomycin, and fetal bovine serum (FBS) were supplied by Invitrogen (Thermo Fisher Scientific, Waltham, MA). Sodium carbonate was obtained from Fisher Scientific (Pittsburgh, PA). High Performance Liquid Chromatography -grade methanol (MeOH) and 95% ethanol were purchased from QRëC Reagent Chemical. Purified water was prepared using a Barnstead E-Pure apparatus. The single-use syringe filter (0.45and 0.2 µm) was purchased from Sartorius, Malaysia. *Andrographis paniculata* dried and ground was purchased from Herba Bagus Sdn. Bhd., Johor, Malaysia. The dried leaves were cooled in cold storage to prevent microbial growth.

Andrographis paniculata leaf extraction

The dried leaves (200 g) of *A. paniculata* were deposited at Forest Research Institute Malaysia, Kuala Lumpur, Malaysia (SBID 002/12). The *A. paniculata* plant was cultivated in a climate-controlled area and air-dried at room temperature. The dried leaves were ground into powder form and passed through a 30-mesh sieve. Extraction was conducted using Dionex™ ASE™ 100 (Thermo Fisher, Waltham, MA). Briefly, 3 g of the sample was mixed with diatomite to remove the remaining moisture before extraction. The obtained sample was then packed into a 10 ml stainless steel vessel extracted with ethanol (99%) at 1,500 psi (10 MPa). The extraction was performed under extraction conditions at a temperature of 60°C under a cycle number of 3 for 5 minutes extraction time. Upon completion, the excess solvent was evaporated using a rotary evaporator (Heidolph MX07R−20, PolyScience, USA), and the extraction liquid sample was oven-dried to a constant weight. The extracts were dissolved in DMSO for further experiments.

Cell culture

The B16F1 melanoma cell line was preserved in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. The cells were incubated at 37°C in a 5% CO_2 incubator.

α-MSH and *A. paniculata* leaf extract treatment

The B16F1 melanoma cells were seeded in six-well plates at a density of 1.5×10^6 cells per well. The wells were supplemented with FBS (10%) and penicillin–streptomycin (1%) in DMEM. The medium was replaced with a fresh medium after 24 hours incubation. This medium was boosted with α-MSH of a 5 nM concentration, and the *A. paniculata* leaf extract at different concentrations (100, 50, 25, 12.5, 6.25, and 3.13 µg/ml) was prepared using this medium. The treatment was done under incubation for about 48 hours. The DMEM solution only was used as the negative control in this treatment. The positive controls, α-MSH (5 nM), arbutin (2 mM), and kojic acid (800 µM), were used comparably with the treatment of the *A. paniculata* leaf extract.

Cell viability assay

Cell viability using the MTT colorimeter assay as described by Hamid *et al.* (2012) was followed with minor modifications to determine the viability of the B16F1 melanoma cells using the *A. paniculata* leaf extract. After incubation for 48 hours, the medium was replaced by the MTT solution and incubated for 90 minutes at 37°C. Then, the solution was substituted with an isopropyl alcohol hydrochloride solution and incubated for 30 minutes at room temperature. The solution was collected and centrifuged for 5 minutes at 13,000 rpm. The supernatant solution was harvested, and the absorbance was determined using an ELISA plate reader at 570 nm. The values were analyzed and compared with the control cells.

Mushroom TYR inhibition assay

An *in vitro* mushroom TYR inhibition assay was carried out as previously described by Qiao *et al.* (2012) and Han *et al.* (2015), with moderate modifications. About 140 µl of the *A. paniculata* leaf extract at a proper concentration was made. A sodium phosphate buffer (pH 6.8) at 10 mM was used in sample preparation and was transferred to a 96-well plate. Approximately 40 µl was poured into each well of 10 µg/ml mushroom TYR in the phosphate buffer at a concentration of 10 mM. The 96 wells were then incubated for about 10 minutes at room temperature. The sample was then added with approximately 20 µl of 10 mM L-DOPA in the phosphate buffer at a concentration of 10 mM. The sample in the 96 wells was incubated for 30 minutes. Absorbance reading was determined using a microplate reader (PerkinElmer, Waltham, MA) at 405 nm. The activity of TYR (%) was determined using

$$\text{Activity of mushroom TYR (\%)} = \frac{C - A}{B} \times 100, \quad (1)$$

where A is the absorbance reading of the sodium phosphate buffer for the untreated mushroom TYR with each sample, B is the absorbance reading of the sodium phosphate buffer for the untreated mushroom TYR with no sample, and C is the absorbance reading of the sodium phosphate buffer for the treated mushroom TYR with each sample.

The calibration curve's linearity range of kojic acid was prepared between 0.00 and 90.00 µg/ml (Figure S1). All absorbance values were measured at 405 nm. Estimating the IC_{50} values for mushroom TYR inhibition (%) of the *A. paniculata* leaf extract used the nonlinear regression plot derived from the plotted data using GraphPad Prism version 9.0.0 for Windows, GraphPad software.

Secreted melanin assay

The secreted melanin assay was carried out as previously described by Kim *et al.* (2016), with slight modifications, to determine the melanin secretion for the *A. paniculata* leaf extract on the B16F1 melanoma cells. The medium of the culture solution was collected and centrifuged for 10 minutes at 10,000 rpm. The absorbance was determined using a microplate reader (PerkinElmer) at 405 nm.

The activity of the secreted melanin (%) was determined using

$$\text{Activity of secreted melanin (\%)} = \frac{C - A}{B} \times 100, \quad (2)$$

where A is the absorbance reading prior to incubation for the medium supplemented with each sample, B is the absorbance reading after 48 hours of incubation for the untreated medium with

each sample, and C is the absorbance reading after 48 hours of incubation for the medium supplemented with each sample.

Intracellular melanin assay

The intracellular melanin assay was carried out as previously reported by Oh *et al*. (2011), with slight modifications. After 48 hours of cell culturing, the cells were harvested and washed twice with potassium-buffered saline (PBS) and lysed with 200 µL of trypsin. Cell pellets were obtained from centrifugation at 1,000 rpm for 10 minutes and lysed with 1 ml of a water: 1N NaOH: DMSO = 7:2:1 solution for 24 hours at 37°C to solubilize the melanin, and 200 µl portions of cell fractions were transferred to 96-well plates. The absorbance of the supernatant was determined by using an ELISA plate reader at 405 nm.

DOPA staining (TYR zymography)

The L-DOPA staining, TYR zymography, assay was performed after 48 hours of cell incubation as previously reported by Lin *et al*. (2011) and Di Petrillo *et al*. (2016), with slight modifications. PBS was used to wash the cultured cells. The radioimmunoprecipitation assay (RIPA) lysis buffer was supplemented with an inhibitor of protease and used to harvest the cultured cells. Similar to the intracellular TYR assay, the amount of protein was also equilibrated in this analysis. Every sample was homogenized using a zymogram sample buffer. Each sample was filled into an 8% gel of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the clear gel was then immersed two times for about 30 minutes in a sodium phosphate buffer at a concentration of 0.1 M. Then, the gel stained in the buffer was added with 0.1 M L-DOPA at 37°C for 1 hour. The dark melanin-containing band was visualized as the TYR activity of the sample.

Western blotting

Western blotting analysis was performed as previously described by Sato *et al*. (2011), with moderate modifications. The B16F1 melanoma cells were seeded in six-well plates at a density of 1.5×10^6 cells per well. The wells were supplemented with FBS (10%) and penicillin–streptomycin (1%) in DMEM. The medium was replaced with a fresh medium after 24 hours incubation. The new medium was added with α-MSH (5 nM), and the *A. paniculata* leaf extract at different concentrations (100, 50, 25, 12.5, 6.25, and 3.13 µg/ml) was prepared using this medium. The treatment was done under 48 hours incubation. The DMEM solution was used as the negative control. The positive controls, *α*-MSH (5 nM), arbutin (2 mM), and kojic acid (800 µM), were used comparably with the treatment of the *A. paniculata* leaf extract. PBS was used to wash the cultured cells. The RIPA lysis buffer supplemented with an inhibitor of protease was used to harvest the cultured cells.

Next, the cells were incubated at 4°C for about 20 minutes. Then, the cell lysates were harvested and centrifuged at 12,000 rpm for about 10 minutes. The supernatant solution was harvested, and the protein concentrations were quantified using the Bradford assay. About 20 µg of protein in each cell lysate sample was boiled for 5 minutes at 95°C in a Laemmli loading buffer (4% SDS, 10% 2-mercaptoethanol, 0.125 M Tris-HCl, 0.2% bromophenol blue, and 0.125 M Tris-HCl). All the ratios for the samples and loading buffer were 1:1. Then, gel electrophoresis of these samples was carried out. The gel was transferred to a polyvinylidene difluoride membrane. A Tris-buffered saline-Tween 20 solution (TBST) containing 5% nonfat dry milk was used to block the membranes. The expression bands of primary antibodies, MITF (60 kDa), TYR (75 kDa), and TRP-2 (55 kDa), were detected in this membrane, respectively, with the rabbit polyclonal MITF antibody (dilution 1:1,000), rabbit polyclonal TYR antibody (dilution 1:1,000), and rabbit polyclonal TRP-2 antibody (dilution 1:1,000). The membrane was incubated with primary antibodies for 24 hours at 4°C. Next, the membrane was washed with TBST and incubated with secondary antibodies, horseradish peroxidase-conjugated anti-rabbit IgG (dilution 1:1,000), for 1 hour at room temperature. The immunoblot results were visualized using an enhanced chemiluminescence solution system (PerkinElmer). A loading control, β-actin (43 kDa), was assessed in this assay.

Statistical analysis

Student's *t*-test was used for statistical significance. The results were interpreted as the mean ± SD of all the data from the replicated experiments.

RESULTS AND DISCUSSION

Cytotoxicity on B16F1 melanoma cells by *A. paniculata* leaf extract

The cytotoxicity assay by the *A. paniculata* leaf extract was investigated on the B16F1 melanoma cells by treating with different concentrations of the *A. paniculata* leaf extract in the presence of α-MSH. The MTT assay was used to determine the cytotoxicity effect of the *A. paniculata* leaf extract on the B16F1 cells. The MTT assay results revealed that the *A. paniculata* leaf extract at a concentration below 50 µg/ml in the presence of α-MSH had no significant effect on cell viability (Fig. 1). Consequently,

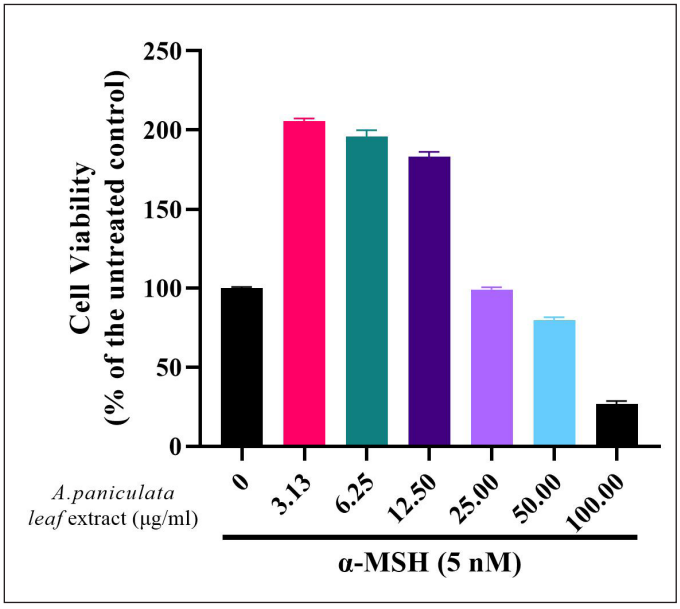

Figure 1. Cell viability effect of *A. paniculata* leaf extract on B16F1 melanoma cells was analyzed by MTT assay. Cells were treated with *A. paniculata* leaf extract and α-MSH (5 nM) simultaneously.

the *A. paniculata* leaf extract at concentrations of 25, 12.5, 6.25, and 3.13 µg/ml was used for future experiments.

Mushroom TYR activity inhibition by *A. paniculata* leaf extract

The *A. paniculata* leaf extract was applied in the mushroom TYR inhibition assay to establish the TYR activity inhibitory effect. The ideal standard used in this assay was kojic acid, known as a recognized inhibitor (Fig. 2). The *A. paniculata* leaf extract showed inhibition in the TYR enzyme at 50 µg/ml of about 74.361% ± 0.026% (Table 1). This result is moderately relative to the standard at 50 µg/ml of kojic acid of about 76.193% ± 0.005% TYR inhibition. This finding indicated that the *A. paniculata* leaf extract has the ability to inhibit TYR activity and melanin synthesis. The main structures of the inhibitor similar to DOPA or tyrosine are from a phenol or catechol derivative (Panzella and Napolitano, 2019). The *A. paniculata* leaf extract has the potential to mimic the amino acid tyrosine extract with the catalytic site binding of the TYR enzyme that blocks pigment formation in skin cells as this study showed inhibition in TYR activity. Therefore, the *A. paniculata* leaf extract can reduce melanogenesis activity.

Andrographis paniculata leaf extract inhibition effect in B16F1 melanoma cells on melanin synthesis

The essential factor in increasing the production of melanin and regulating melanogenesis is α-MSH (Buscà and Ballotti, 2000). Hence, this study evaluated the *A. paniculata* leaf extract on melanin inhibition in the α-MSH-induced B16F1 melanoma cells. The secreted melanin content elevated twofold once with α-MSH treatment, as in Figure 3. Simultaneously, the *A. paniculata* leaf extract had a morphological outcome on the B16F1 melanoma cells, as shown in Figure 4. Melanin secretion reduction comparable with the untreated control was demonstrated by arbutin, indicating its impressive antimelanogenic activity. In the meantime, remarkable reduction of melanin secretion was also shown by kojic acid. The spectacular inhibitory effect of the *A. paniculata* leaf extract on melanin secretion at 25 µg/ml was more powerful than that of kojic acid and arbutin in this condition of the experiment. The *A. paniculata* leaf extract was shown to dose-dependently reduce melanin content secretion. The powerful inhibitory effect on melanin secretion at 25 µg/ml indicated potency reduction was more effective than that of arbutin (2 and 4 mM) and kojic acid (400 and 800 µM).

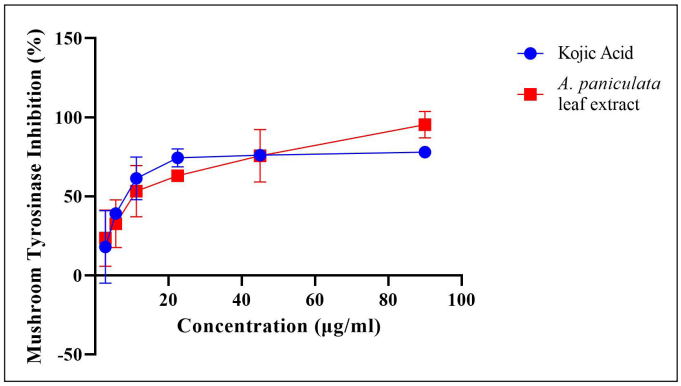

Figure 2. Mushroom tyrosinase activity by *A. paniculata* leaf extract.

Intracellular TYR activity reduced by *A. paniculata* leaf extract

TYR direct inhibition or also the expression of gene suppression encouraged the reduction of the protein level in skin cells. This can be established by TYR activity reduction in melanocytes. In the prior experiment, the *A. paniculata* leaf extract had an outstanding TYR inhibitory activity. Next, the experiment to determine the effect on the intracellular TYR activity of the *A. paniculata* leaf extract was examined. Cell lysates were obtained from the B16F1 melanoma cells treated with different concentrations of the *A. paniculata* leaf extract. This was used as the source of TYR. The key enzyme that manages melanin pigment formation in humans and animals is TYR (Han *et al*., 2015). Then, the B16F1 melanoma cells were treated in the presence of α-MSH with different concentrations of the *A. paniculata* leaf extract, arbutin (2 and 4 mM), and kojic acid (400 and 800 µM). Afterward, TYR activity was measured. From the results, the *A. paniculata* leaf extract was inhibited and showed a concentration-dependent behavior as in the α-MSH-induced melanocytes by the activity of the TYR enzyme. This experiment exhibited that TYR enzyme activity inhibition by the *A. paniculata* leaf extract was more significant than that by arbutin (2 and 4 mM) and kojic acid (400 and 800 µM), as shown in Figure 5. T TYR activity was suppressed by 45.4% at a concentration of 25 µg/ml of the *A. paniculata* leaf extract.

Table 1. Tyrosinase inhibition of *A. paniculata* leaf extract in mushroom TYR activity. Kojic acid was used as a positive control.

Compound	Concentration (µg/ml)	Tyrosinase inhibition(%)
Kojic acid	50	76.193 ± 0.0055
Andrographis. paniculata leaf extract	50	74.361 ± 0.026

TYR zymography of *A. paniculata* leaf extract showed positive suppresive effect on the intracellular TYR activity

TYR activity assessment was done after SDS-PAGE protein separation. Molecular weight was used as the basis for TYR separation on a gel. The gel could demonstrate its enzymatic process in the order that it could oxidize the L-DOPA solution when exerted on the gel. Therefore, the dark color of DOPA quinone will be formed (Sato *et al*., 2008). TYR zymography was carried out with the purpose of identifying TYR activity. The *A. paniculata* leaf extract showed its role in TYR degradation of the α-MSH-activated B16F1 melanoma cells. TYR activity was suppressed initially at the concentration of 6.25 µg/ml (Fig. 6). Nearly none of the activated TYR remained at the concentration of 25 µg/ml. The results showed highly demonstrated intracellular TYR comparable with TYR zymography. As shown, the result suggested that at the concentration of 25 µg/ml the *A. paniculata* leaf extract is equivalent to arbutin (2 and 4 mM), which is a known great TYR inhibitor.

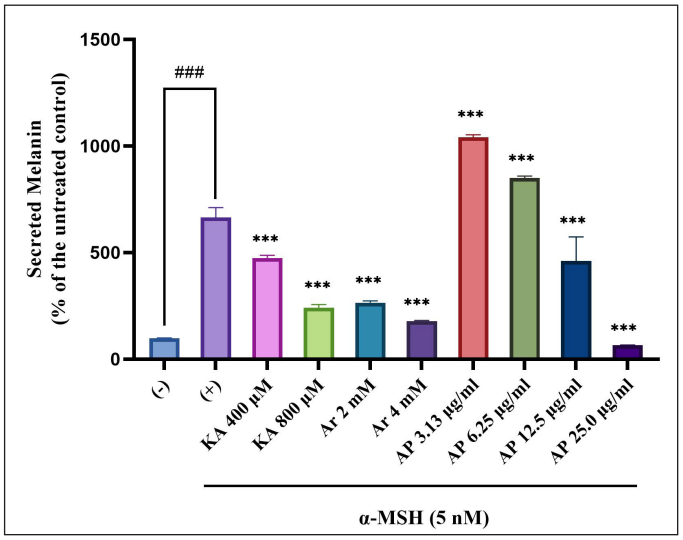

Figure 3. Melanin production inhibition effect in melanocytes of *A. paniculata* leaf extract. The results represent the mean ± SD of triplicate samples. ###Statistically significant ($p < 0.001$) compared to control group. ***Statistically significant ($p < 0.001$) compared to α-MSH group.

Immunoblot showed that *A. paniculata* leaf extract downregulated TYR, TRP-2, and MITF expressions

To evaluate the *A. paniculata* leaf extract in regulating the protein expression of melanogenic enzymes like TYR, immunoblotting or Western blot analysis was carried out using different concentrations of the *A. paniculata* leaf extract. The loading control used in this study was β-actin. Intracellular TYR activity intensification for similar conditions corresponded to this, as the result showed when treated with α-MSH at the 75 kDa TYR enhancement marker. Treatment induced by α-MSH showed that the level of TYR protein was elevated and the induction was inhibited significantly by the *A. paniculata* leaf extract, as shown in Figure 7. The inhibition relatively approached at 6.25 µg/ml, 12.5 µg/ml and 25 µg/ml The regulation possibly exerted at TYR gene expression level and *A. paniculata* leaf extract was demonstrated depleted the intracellular TYR activity. The result showed that TYR protein decreased similarly to kojic acid (800 µM) starting at 6.25 until 25 µg/ml treatment of the *A. paniculata* leaf extract. TYR was presented as a crucial enzyme in ideal melanin production (Zhu *et al*., 2015).

The effects of the *A. paniculata* leaf extract on the protein expression level of TRP-2 were evaluated by western blot analysis.

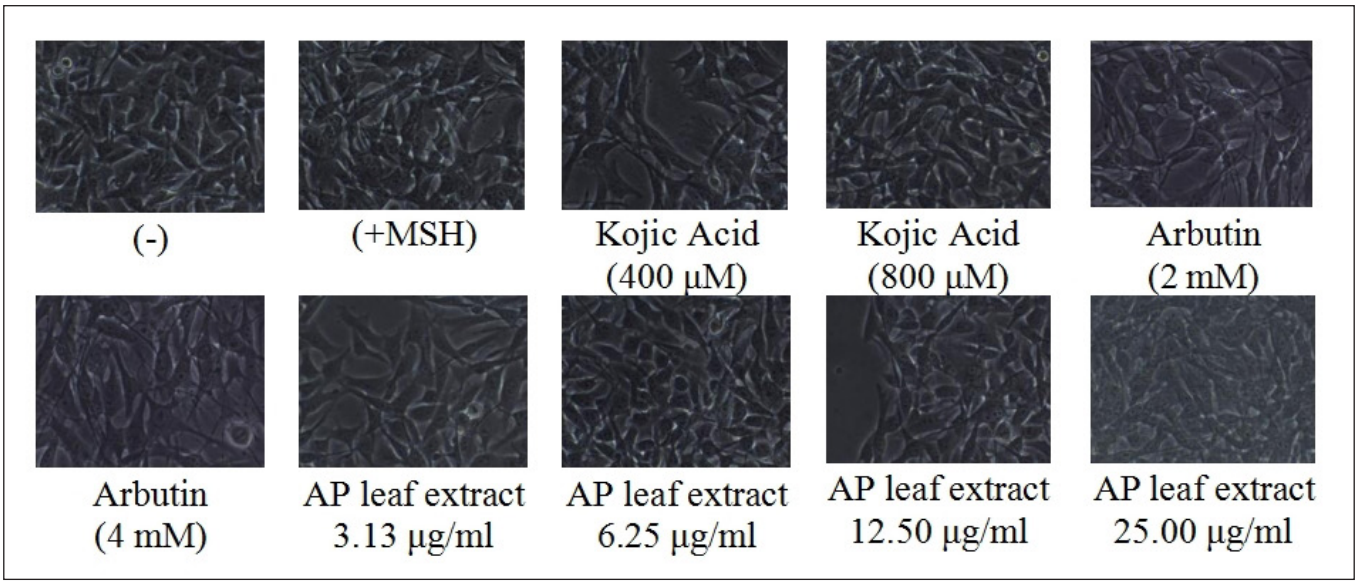

Figure 4. Representative morphology of B16F1 melanoma cells from Figure 3 by an inverted phase-contrast microscope.

The result found that the *A. paniculata* leaf extract suppressed the expression level of TRP-2 slightly approached at 3.13 µg/ml and 6.25 µg/ml simultaneously in the B16F1 melanoma cells stimulated by α-MSH (Fig. 8). In the meantime, the *A. paniculata* leaf extract significantly suppressed TRP-2 at 25 µg/ml.

MITF is the main component in the regulation of tyrosinase-related proteins which incorporate TRP-1 and TRP-2. The regulation happens throughout the melanogenesis process in mammalian cells. As MITF is the main transcription factor in modulating the expression of the major melanogenic genes, the effectiveness of the *A. paniculata* leaf extract was evaluated on MITF expression. The result, as shown in Figure 9, demonstrated that the *A. paniculata* leaf extract significantly reduced the MITF expression level, which indicated that the *A. paniculata* leaf extract had significant inhibition activity towards MITF protein expression. In western blot analysis, in Figure 9, MITF was downregulated in a dose-dependent manner and significantly reduced at 25 µg/ml.

As prior studies expressed, TYR and TRP-2 are incorporated into the pathway of melanogenesis for moderate essential responses to melanogenesis (Jin *et al*., 2014; Xue *et al*., 2018). The hypopigmentation effect of the *A. paniculata* leaf extract as possibly the outcome of MITF gene expression was downregulated and suppressed the gene and protein expression of TYR, TRP-1, and TRP-2.

A copper-containing enzyme, TYR, is the major component in the control of melanin production (Lajis *et al*., 2012; Zolghadri *et al*., 2019). Therefore, hyperpigmentation can be treated by a compound that can control TYR and activity (Briganti *et al*., 2003; Lim *et al*., 2019). Moreover, previous studies reported melanin production and hyperpigmentation are elevated as the

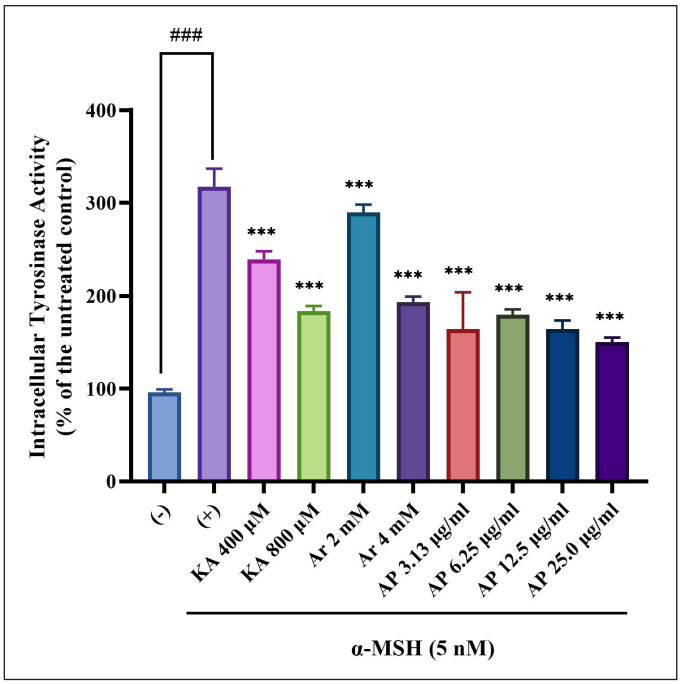

Figure 5. Intracellular tyrosinase activity of B16F1 melanoma was inactivated by *A. paniculata* leaf extract. Intracellular tyrosinase activity reduction was discovered. The results represent the mean ± SD of triplicate samples. ### Statistically significant ($p < 0.001$) compared to control group. ***Statistically significant ($p < 0.001$) compared to α-MSH group.

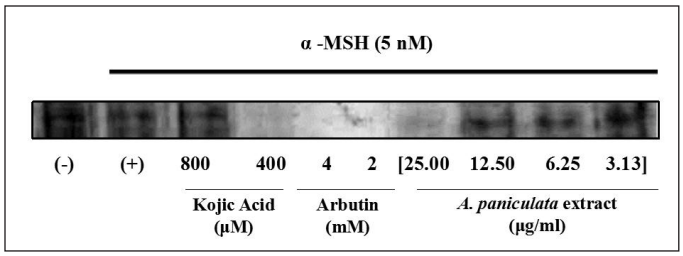

Figure 6. Decreased tyrosinase activity by A. paniculata leaf extract confirmed through the result of tyrosinase zymography.

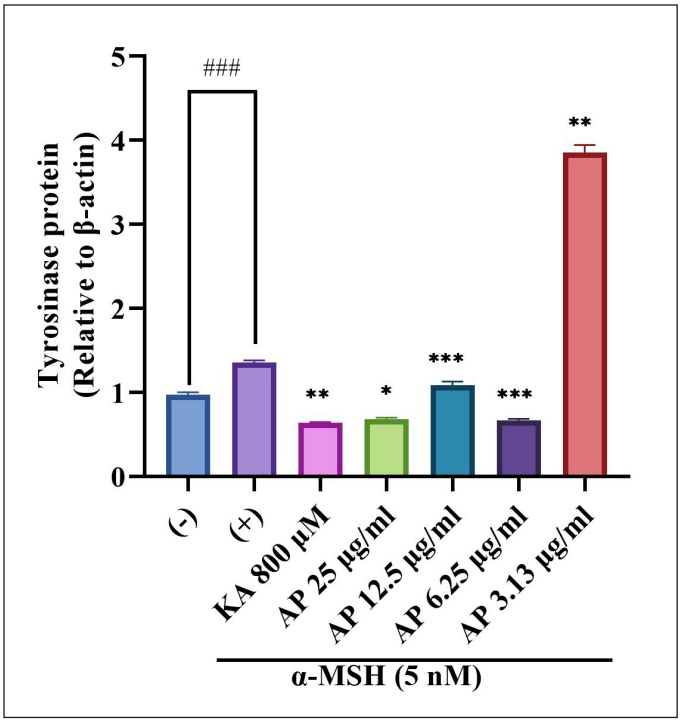

Figure 7. Effects of *A. paniculata* leaf extract in α-MSH-induced B16F1 melanoma cells on expression levels of tyrosinase were analyzed by different concentrations of *Andrographis paniculata* leaf extract (AP; 3.13, 6.25, 12.50, and 25.00 µg/ml) or kojic acid (KA; 800 µM) for about 48 hours in α-MSH presence. The results represent the mean ± SD of triplicate samples. ### Statistically significant ($p < 0.001$) compared to control group. Statistically significant at ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ compared to α-MSH group.

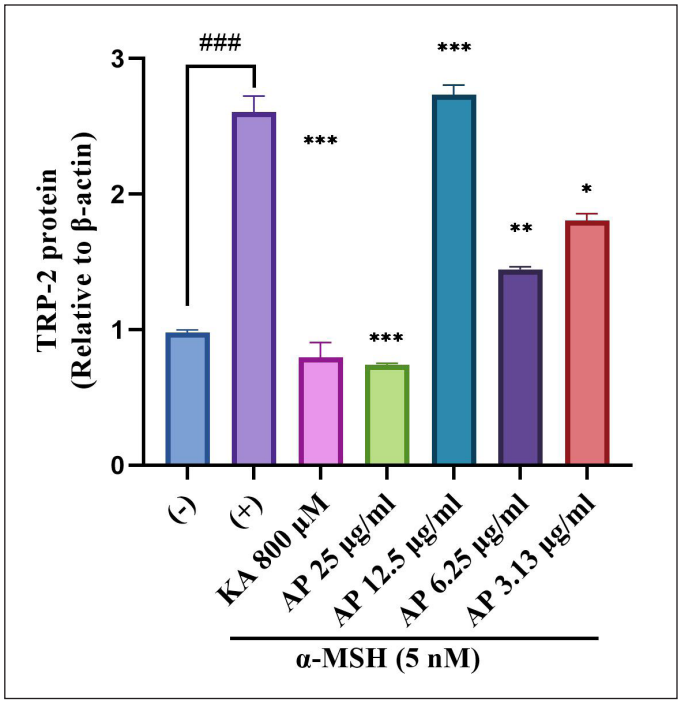

Figure 8. Effects of *A. paniculata* leaf extract in α-MSH-induced B16F1 melanoma cells on expression levels of TRP-2 were analyzed by different concentrations of *A. paniculata* leaf extract (3.13, 6.25, 12.50, and 25.00 µg/ml) or kojic acid at 800 µM and arbutin at 2 mM for about 48 hours in α-MSH presence. The results represent the mean ± SD of triplicate samples. ### Statistically significant ($p < 0.001$) compared to control group. Statistically significant at ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ compared to α-MSH group.

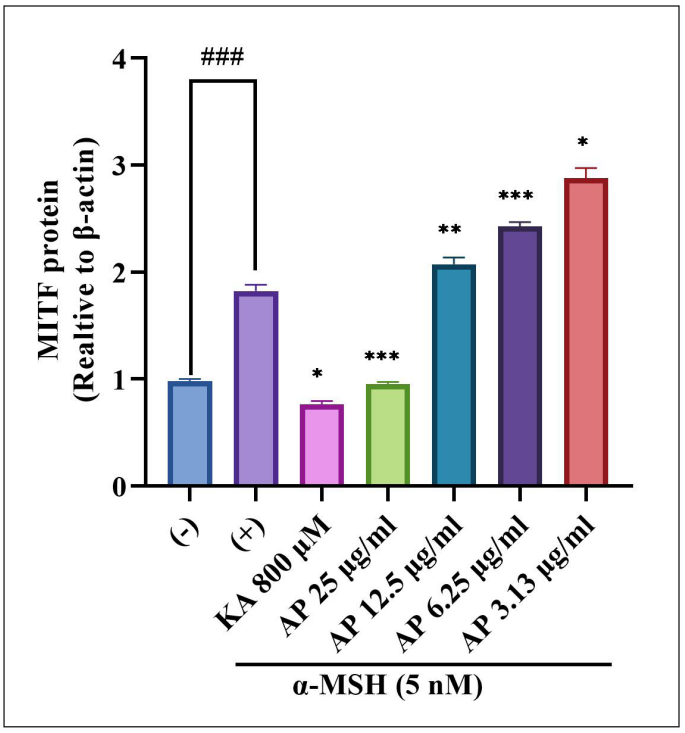

Figure 9. Effects of *A. paniculata* leaf extract in α-MSH-induced B16F1 melanoma cells on expression levels of MITF were analyzed by different concentrations of *A. paniculata* leaf extract (3.13, 6.25, 12.50, and 25.00 µg/ml) or kojic acid at 800 µM and arbutin at 2 mM for about 48 hours in α-MSH presence. The results represent the mean ± SD of triplicate samples. ### Statistically significant ($p < 0.001$) compared to control group. Statistically significant at ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ compared to α-MSH group.

generation of *ROS* increases (Kamiński *et al*., 2022; Qiao *et al*., 2012). Hence, this study was conducted using the *A. paniculata* leaf extract known for its potency in antioxidant activity. The *A. paniculata* leaf extract exhibited an antimelanogenic effect in a concentration-dependent mode based on the result of this study. This antimelanogenic possession was applied without particularly affecting the cytotoxicity of the B16F1 melanoma cells.

Furthermore, there was a study that found andrographolide in *A. paniculata*-induced apoptosis in HT-29 cells, which seemed to be linked with the augmented intracellular *ROS* level (Khan *et al*., 2018). MITF engagement in regulating genes such as TYR, TRP1 and TRP 2 expression as MITF main transcription factor in melanogenic activity (Kim *et al*., 2013; Villareal *et al*., 2017). In this state, it plays crucial roles in melanin derivation from tyrosine.

CONCLUSION

The *A. paniculata* leaf extract suppressed the enzyme expression of TYR and MITF in a concentration-dependent mode. The results from our study indicated the significantly reduced protein expression of TRP-2 in certain concentration conditions. It can suppress melanin production. Thus, the *A. paniculata* leaf extract at 25 µg/ml is highly possibly applicable being a powerful natural and harmless ingredient as a skin lightening agent as the extract possesses a melanin production inhibition property. The *A. paniculata* leaf extract could represent novelty for antimelanogenesis potential through TYR and MITF and its related protein inhibitions. The *A. paniculata* leaf extract is safe and reliable for application as an antimelanogenic agent for hyperpigmentation control.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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REFERENCES

Bello OA, Ayanda OI, Aworunse OS, Olukanmi BI. Exploring the mechanism of cytotoxic and anti-inflammatory property of andrographolide and its derivatives. Pharmacogn Rev, 2018; 1:56–65.

Bhattar PA, Zawar VP, Godse KV, Patil SP, Nadkarni NJ, Gautam MM. Exogenous ochronosis. Indian J Dermatol, 2015; 60(6):537–43.

Briganti S, Camera E, Picardo M. Chemical and instrumental approaches to treat hyperpigmentation. Pigment Cell Res, 2003; 16:101–10.

Buscà R, Ballotti R. Cyclic AMP a key messenger in the regulation of skin pigmentation. Pigment Cell Res, 2000; 13(2):60–9.

Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev, 2011; 75(1):50–83.

Chang MS, Choi MJ, Park SY, Park SK. Inhibitory effects of hoelen extract on melanogenesis in B16F1 melanoma cells. Phytother Res, 2010; 24:1359–64.

Chao WW, Kuo YH, Lin BF. Isolation and identification of *Andrographis paniculata* (Chuanxinlian) and its biologically active constituents inhibited enterovirus 71-induced cell apoptosis. Front Pharmacol, 2021; 12:1–11.

Denat L, Kadekaro AL, Marrot L, Leachman SA, Abdel-Malek ZA. Melanocytes as instigators and victims of oxidative stress. J Invest Dermatol, 2014; 134(6):1512–8.

Di Petrillo A, González-Paramás AM, Era B, Medda R, Pintus F, Santos-Buelga C, Fais A. Tyrosinase inhibition and antioxidant properties of *Asphodelus microcarpus* extracts. BMC Complement Altern Med, 2016; 16:1–9.

dos santos Videira IF, Moura DFL, Magina S. Mechanisms regulating melanogenesis. An Bras Dermatol, 2013; 88:76–83.

Hamid MA, Sarmidi MR, Park CS. Mangosteen leaf extract increases melanogenesis in B16F1 melanoma cells by stimulating tyrosinase activity in vitro and by up-regulating tyrosinase gene expression. Int J Mol Med, 2012; 29:209–17.

Han SM, Kim JM, Pak SC. Anti-melanogenic properties of honeybee (*Apis mellifera* L.) venom in α-MSH-stimulated B16F1 cells. Food Agric Immunol, 2015; 26:451–62.

Hossain S, Urbi Z, Karuniawati H, Mohiuddin RB, Moh Qrimida A, Allzrag AMM, Ming LC, Pagano E, Capasso R. *Andrographis paniculata* (Burm. f.) Wall. ex Nees: an updated review of phytochemistry, antimicrobial pharmacology, and clinical safety and efficacy. Life (Basel), 2021; 11(4):348.

Hu Frisk JMH, Kjellén L, Melo FR, Öhrvik H, Pejler G. Mitogen-activated protein kinase signaling regulates proteoglycan composition of mast cell secretory granules. Front Immunol, 2018; 9:1670.

Jeon NJ, Kim YS, Kim EK, Dong X, Lee JW, Park JS, Shin WB, Moon SH, Jeon BT, Park PJ. Inhibitory effect of carvacrol on melanin synthesis via suppression of tyrosinase expression. J Funct Foods, 2018; 45:199–205.

Jin KS, Oh YN, Hyun SK, Kwon HJ, Kim BW. Betulinic acid isolated from *Vitis amurensis* root inhibits 3-isobutyl-1-methylxanthine induced melanogenesis via the regulation of MEK/ERK and PI3K/Akt pathways in B16F10 cells. Food Chem Toxicol, 2014; 68:38–43.

Kamiński K, Kazimierczak U, Kolenda T. Oxidative stress in melanogenesis and melanoma development. Contemp Oncol (Pozn), 2022; 26(1):1–7.

Kawakami A, Fisher DE. The master role of microphthalmia-associated transcription factor in melanocyte and melanoma biology. Lab Invest, 2017; 97:649–56.

Khan I, Khan F, Farooqui A, Ansari IA. Andrographolide exhibits anticancer potential against human colon cancer cells by inducing cell cycle arrest and programmed cell death via augmentation of intracellular reactive oxygen species level. Nutr Cancer, 2018; 70(5):787–803.

Kim MJ, Jung TK, Park H, Yoon K. Effect of hoechunyangkyeok-san extract on melanogenesis. J Cosmet Dermatol Sci Appl, 2016; 6:85–95.

Kim SS, Kim MJ, Choi YH, Kim BK, Kim KS, Park KJ, Park SM, Lee NH, Hyun CG. Down-regulation of tyrosinase, TRP-1, TRP-2 and MITF expressions by citrus press-cakes in murine B16 F10 melanoma. Asian Pac J Trop Biomed, 2013; 3(8):617–22.

Lajis AFB, Hamid M, Ariff AB. Depigmenting effect of kojic acid esters in hyperpigmented B16F1 melanoma cells. J Biomed Biotechnol, 2012; 2012:952452.

Lee AY. Skin pigmentation abnormalities and their possible relationship with skin aging. Int J Mol Sci, 2021; 22(7):3727.

Lim J, Nam S, Jeong JH, Kim MJ, Yang Y, Lee MS, Lee HG, Ryu JH, Lim JS. Kazinol U inhibits melanogenesis through the inhibition of tyrosinase-related proteins via AMP kinase activation. Br J Pharmacol, 2019; 176(5):737–50.

Lin VC, Ding HY, Tsai PC, Wu JY, Lu YH, Chang TS. In vitro and in vivo melanogenesis inhibition by biochanin a from *Trifolium pratense*. Biosci Biotechnol Biochem, 2011; 75:914–8.

Lin HC, Su SL, Lu CY, Lin AH, Lin WC, Liu CS, Yang YC, Wang HM, Lii CK, Chen HW. Andrographolide inhibits hypoxia-induced HIF-1α-driven endothelin 1 secretion by activating Nrf2/HO-1 and promoting the expression of prolyl hydroxylases 2/3 in human endothelial cells. Environ Toxicol, 2017; 32(3):918–30.

Liu YT, Chen HW, Lii CK, Jhuang JH, Huang CS, Li ML, Yao HT. A diterpenoid, 14-deoxy-11, 12-didehydroandrographolide, in *Andrographis* p*aniculata* reduces steatohepatitis and liver injury in mice fed a high-fat and high-cholesterol diet. Nutrient, 2020; 12(2):523.

Loureiro Damasceno JPL, Silva da Rosa H, Silva de Araújo L, Furtado NAJC. *Andrographis paniculata* formulations: impact on diterpene lactone oral bioavailability. Eur J Drug Metab Pharmacokinet, 2022; 47(1):19–30.

Munshi A, Ramesh R. Mitogen-activated protein kinases and their role in radiation response. Genes Cancer, 2013; 4(9–10):401–8.

Ngeow KC, Friedrichsen HJ, Li L, Zeng Z, Andrews S, Volpon L, Brunsdon H, Berridge G, Picaud S, Fischer R, Lisle R, Knapp S, Filippakopoulos P, Knowles H, Steingrímsson E, Borden KLB, Patton EE, Goding CR. BRAF/MAPK and GSK3 signaling converges to control MITF nuclear export. In: Proceedings of the National Academy of Sciences of the United States of America, 2018; 115:E8668–77.

Oh E, Kim HJ, Lee D, Kang JH, Kim HG, Han SH, Baek NI, Kim KT. 8-Methoxybutin inhibits α-MSH induced melanogenesis and proliferation of skin melanoma by suppression of the transactivation activity of microphthalmia-associated transcription factor. Biomed Pharmacother, 2022; 152:113272.

Oh MJ, Hamid MA, Ngadiran S, Seo YK, Sarmidi MR, Park CS. *Ficus deltoidea* (Mas cotek) extract exerted anti-melanogenic activity by preventing tyrosinase activity in vitro and by suppressing tyrosinase gene expression in B16F1 melanoma cells. Arch Dermatol Res, 2011; 303:161–70.

Olea-Flores M, Zuñiga-Eulogio MD, Mendoza-Catalán MA, Rodríguez-Ruiz HA, Castañeda-Saucedo E, Ortuño-Pineda C, Padilla-Benavides T, Navarro-Tito N. Extracellular-signal regulated kinase: a central molecule driving epithelial-mesenchymal transition in cancer. Int J Mol Sci, 2019; 20(12):2885.

Panzella L, Napolitano A. Natural and bioinspired phenolic compounds as tyrosinase inhibitors for the treatment of skin hyperpigmentation: recent advances. *Cosmetics,* 2019; 6(4):57.

Paruchuru LB, Govindaraj S, Razin E. The critical role played by mitochondrial MITF serine 73 phosphorylation in immunologically activated mast cells. Cells, 2022; 11(3):589.

Pfisterer PH, Rollinger JM, Schyschka L, Rudy A, Vollmar AM, Stuppner H. Neoandrographolide from *Andrographis paniculata* as a potential natural chemosensitizer. *Planta Med, 2010; 76(15):*1698–700.

Pillaiyar T, Manickam M, Namasivayam V. Skin whitening agents: medicinal chemistry perspective of tyrosinase inhibitors. J Enzyme Inhib Med Chem, 2017; 32(1):403–25.

Pop C, Vlase L, Tamas M. Natural resources containing arbutin. determination of arbutin in the leaves of *Bergenia crassifolia* (L.) fritsch. acclimated in Romania. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 2009; 37:129–32.

Qiao Z, Koizumi Y, Zhang M, Natsui M, Flores MJ, Gao L, Yusa K, Koyota S, Sugiyama T. Anti-melanogenesis effect of *Glechoma hederacea* L. extract on B16 murine melanoma cells. Biosci Biotechnol Biochem, 2012; 76(10):1877–83.

Rahmi EP, Kumolosasi E, Jalil J, Buang F, Jamal JA. Extracts of *Andrographis paniculata* (Burm.f.) nees leaves exert anti-gout effects by lowering uric acid levels and reducing monosodium urate crystal-induced inflammation. Front Pharmacol, 2022; 12:787125.

Sato K, Ando R, Kobayashi H, Nishio T. 2-Ethoxybenzamide stimulates melanin synthesis in B16F1 melanoma cells via the CREB signaling pathway. Mol Cell Biochem, 2016; 423:39–52.

Sato K, Morita M, Ichikawa C, Takahashi H, Toriyama M. Depigmenting mechanisms of all-trans retinoic acid and retinol on B16 melanoma cells. Biosci Biotechnol Biochem, 2008; 72:2589–97.

Sato K, Takahashi H, Toriyama M. Depigmenting mechanism of NSAIDs on B16F1 melanoma cells. Arch Dermatol Res, 2011; 303:171–80.

Shu J, Huang R, Tian Y, Liu Y, Zhu R, Shi G. Andrographolide protects against endothelial dysfunction and inflammatory response in rats with coronary heart disease by regulating PPAR and NF-κB signaling pathways. Ann Cardiothorac Surg, 2020; 9:1965–75.

Singh M, Mukhopadhyay K. Alpha-melanocyte stimulating hormone: an emerging anti-inflammatory antimicrobial peptide. Biomed Res Int, 2014; 2014:874610.

Singh N. A review on herbal plants used as immunomodulators. Int J Pharm Res, 2016; 13:3602–10.

Song YS, Balcos MC, Yun HY, Baek KJ, Kwon NS, Kim MK, Kim DS. ERK activation by fucoidan leads to inhibition of melanogenesis in Mel-Ab cells. Korean J Physiol Pharmacol, 2015; 19(1):29–34.

Sun L, Guo C, Yan L, Li H, Sun J, Huo X, Xie X, Hu J. Syntenin regulates melanogenesis via the p38 MAPK pathway. Mol Med Rep, 2020; 22(2):733–8.

Sunkara HP, Kilaru KR, Kumar AP, Ramineni HB, Krishna PR. A case report on hydroquinone induced exogenous ochronosis. Int J Adv Med, 2020; 7:337.

Villareal MO, Kume S, Neffati M, Isoda H. Upregulation of mitf by phenolic compounds-rich *Cymbopogon schoenanthus* treatment promotes melanogenesis in B16 melanoma cells and human epidermal melanocytes. Biomed Res Int, 2017; 2017:8303671.

Xu C, Chou GX, Wang ZT. A new diterpene from the leaves of *Andrographis paniculata* Nees. Fitoterapia, 2010; 81(6):610–3.

Xue L, Li Y, Zhao B, Chen T, Dong Y, Fan R, Li J, Wang H, He X. TRP-2 mediates coat color pigmentation in sheep skin. Mol Med Rep, 2018; 17(4):5869–77.

Zhu PY, Yin WH, Wang MR, Dang YY, Ye XY. Andrographolide suppresses melanin synthesis through Akt/GSK3β/β-catenin signal pathway. J Dermatol Sci, 2015; 79(1):74–83.

Zolghadri S, Bahrami A, Hassan Khan MT, Munoz-Munoz J, Garcia-Molina F, Garcia-Canovas F, Saboury AA. A comprehensive review on tyrosinase inhibitors. J Enzyme Inhib Med Chem, 2019; 34(1):279–309.

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SUPPLEMENTARY MATERIAL

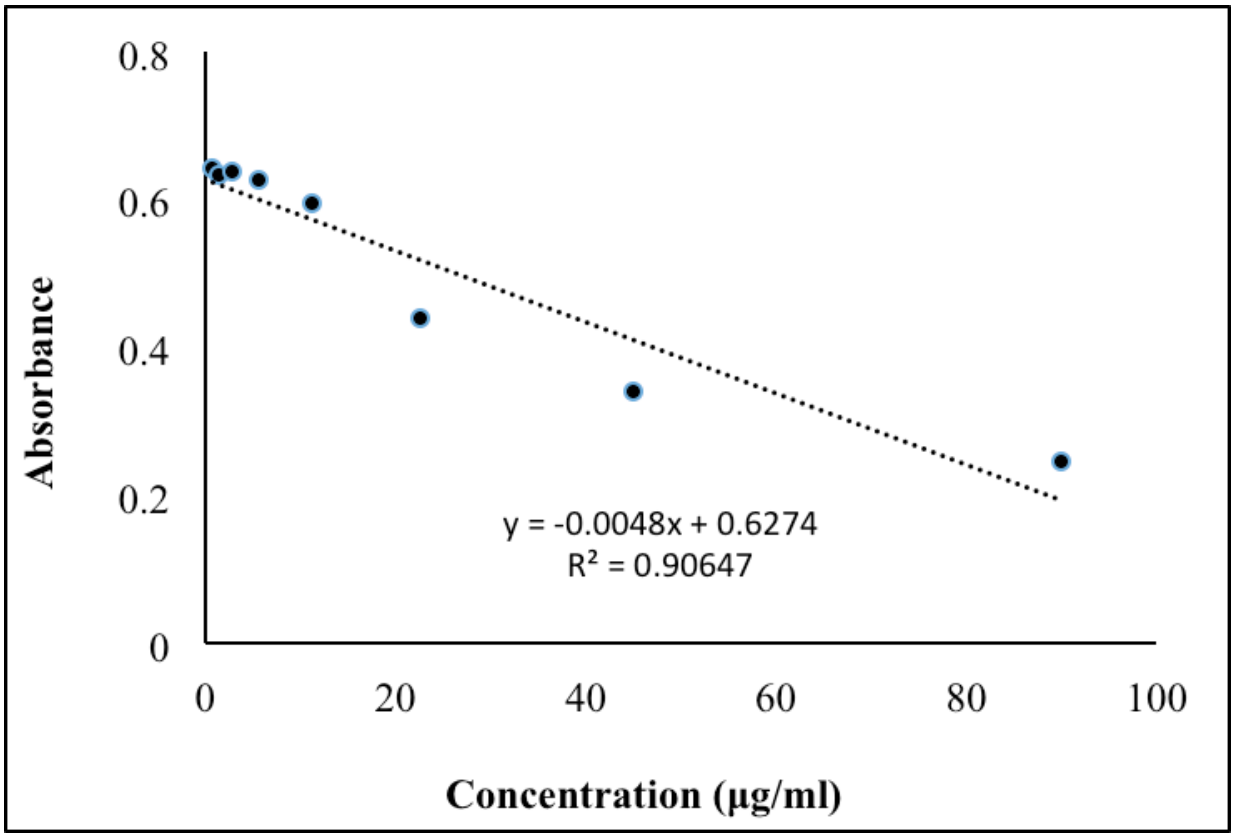

Figure S1. Calibration curve for reference standard of kojic acid for mushroom tyrosinase assay.