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, neandrographolide, 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
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-Melanoctye-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 nonethetically 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 extracelluar signal-regulated protein kinases (ERSPKs), 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, ERSPK 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 ERSPK 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 perusable 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 QRcE 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% CO₂ 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 × 10⁶ 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 nM. 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 5 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 IC₅₀ 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,
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).

**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. TYR activity was suppressed by 45.4% at a concentration of 25 μg/ml of the *A. paniculata* leaf extract.

![Figure 2. Mushroom tyrosinase activity by *A. paniculata* leaf extract.](image)

| 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 suppressive 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.

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