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Inhibition of melanogenesis in murine melanoma cells by *Agaricus brasiliensis* methanol extract and anti-reactive oxygen species (ROS) activity

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***Agaricus brasiliensis* is a valuable food mushroom that exhibits several pharmacological activities. The beneficial effects of the *A. brasiliensis* methanol extract on skin health have never been investigated. The purpose of this study was to evaluate the potential skin care effects of extract from *A. brasiliensis*. The results reveal that *A. brasiliensis* methanol extract exhibited apparent inhibitory effects on melanin synthesis and suppressed the expression levels of melanogenesis-related proteins. Also, the extract depleted the intracellular level of the reactive oxygen species (ROS). The methanol extract inhibited mushromm tyrosinase activity, suppressed intracellular tyrosinase activity (IC_{50} = 0.713 mg/mL) and decreased the amount of melanin (IC_{50} = 0.711 mg/ml) in B16F10 cells. The protein expression levels of tyrosinase and tyrosinase-related protein 1 (TRP 1) were decreased by the methanol extract. Our results show that *A. brasiliensis* methanol extract inhibited melanogenesis and depleted intracelluylar ROS in B16F10 cells. Thus, *A. brasiliensis* methanol extract could be applied as a type of natural skin depigmentinging agent in skin care products.**

Key words: *Agaricus brasiliensis*, tyrosinase, melanin, reactive oxygen species (ROS).

INTRODUCTION

Agaricus brasiliensis, also called *Agaricus blazei* Murill, is a mushroom native to Brazil and is currently popular in the market in Taiwan. It has been reported as a functional health food for its use as a complementary and alternative medicine by cancer patients (Talcott et al., 2007). A broad spectrum of biological activities has been attributed to this mushroom including antibacterial, antiparasitic, antiviral, antitumoral and anti-genotoxic actions. Moreover, the fungus was also effective in treating diabetes and hepatitis in mice model (Martins de Oliveira et al., 2002; Mizuno, 2002). However, there are limited reports on the beneficial effects of *A. brasiliensis* on skin health. Our previous study revealed that aqueous and ethanol extracts of *A. brasiliensis* provide inhibitory effect on mushroom tyrosinase activity (Chien et al., 2008), which suggested that this edible mushroom could be effective in reducing melanin production in melanocytes.

The excess ROS may oxidize cellular components and inhibit their normal physiological function (Valko et al., 2007). Further, the overproduction of reactive oxygen species (ROS) has been reported to be related to the aging processes (Halliwell, 1996). The antioxidant activity of various extract of *A. brasiliensis* has been evaluated by different methods(Huang and Mau, 2006). However, none

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of the reports evaluated the effect of the *A. brasiliensis* extract on intracellular ROS production.

Melanin is responsible for skin color and plays an important role in protection of the skin against UV harm. The abnormal accumulation of melanin could cause several skin disorders such as melasma, age spots and freckles (Briganti et al., 2003). In the melanin biosynthesis pathway, microphthalmia-associated transcriotion factor (MITF), tyrosinase, tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2) contribute to the production of melanin (Hearing and Jimenez, 1987). Furthermore, it is reported that melanogenesis produce hydrogen peroxide (H_2O_2) and other ROS, which put the melanocytes under high-grade oxidative stress. The ROS scavengers and inhibitors of ROS generation may down-regulate UV-induced melanogenesis (Yamakoshi et al., 2003). However, scientific studies on the antimelanogenic properties of *A. brasiliensis* are lacking. In this study, we aimed to examine the inhibitory effects of the *A. brasiliensis* methanol extract on melanogenesis and intra-cellular ROS production in B16F10 melanoma cells.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and solvents were obtained from Sigma-Aldrich (Sigma Chemical Co, Saint Louis, MO, USA).

Preparation of *A. brasiliensis* extract

The mushroom *A. brasiliensis* (*Agaricus blazei* Murill) (ATCC 76739; BCRC 36814) was obtained from the Bioresource Collection and Research Center (BCRC) in Taiwan. Culture of the mushroom was grown in 1,000-mL Erlenmeyer flasks containing 200 mL potato dextrose broth (BD Diagnostics, Spark, MD, USA) and incubated at 25°C for 7 days with 100 rpm shaking. The mycelia were then filtered using Whatman Grade No. 1 filter paper (VWR International, West Chester, PA, USA), lyophilized, and powered before use. Dried mycelium powder (4 g) was extracted with 80% (v/v) methanol at room temperature for three days. The extract was filtrated, pooled, and allowed to evaporate completely in a rotary evaporator at mild temperature (40°C). The dried residue was finally dissolved in dimrthylsulfoxide (DMSO) into different concentrations for further experiments.

Cell culture

B16F10 cells (ATCC CRL-6475; BCRC60031) were cultured in Dulbecco's modification of Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco, Langley, OK, USA) and penicillin/streptomycin (100 I.U/50 µg/mL) (Sigma Chemical Co, Saint Louis, MO, USA) in a humidified atmosphere containing 5% CO_2 in air at 37°C. All the experiments were performed in triplicate and were repeated 3 times to ensure reproducibility.

Cell viability assay

The cell viability assay was performed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Tada, Shiho et al., 1986). The cells (1×10^4 cells/well) were seeded into a 96-well plate and cultured overnight, and then the cells were exposed to various concentrations of *A. brasiliensis* methanol extract (0.1-10 mg/mL) for 24 h followed by the addition of MTT solution to the wells. The insoluble derivative of MTT produced by intracellular dehydrogenase was solubilized with ethanol-DMSO (1:1 mixture solution). The absorbance of each well at 570 nm was read using a microplate reader.

Assay of mushroom tyrosinase activity

The mushroom tyrosinase inhibition experiments were carried out in triplicate as described previously (Huang et al., 2012). The 10 µL of aqueous solution of mushroom tyrosinase (200 units) was added to a 96-well microplate, in a total volume of 200 µL mixture containing 5 mM L-DOPA, 50 mM phosphate buffer (pH 6.8) and the methanol extract or kojic acid (200 µM). The assay mixture was incubated at 37°C for 30 min. After incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 490 nm in a microplate reader.

Measurement of melanin content in B16F10 cells

The intracellular melanin content was measured as previously described (Huang et al., 2012). The B16F10 cells were first stimulated with α-melanocyte stimulating hormone (α-MSH) (100 nM) for 24 h, and then treated with either *A. brasiliensis* methanol extract (0.1, 0.25, 0.5 and 0.75 mg/mL) or arbutin (2.0 mM) for a further 24 h. After treatment, the cells were detached by incubation in trypsin/ethylenediaminetetraacetic acid (EDTA) and subsequently centrifuged at 5,000 µg for 10 min. After precipitation, the cell pellets were solubilized in 1 N NaOH at 60°C for 60 min. The melanin content was assayed by spectrophotometric analysis at 405 nm absorbance.

Assay of B16F10 intracellular tyrosinase activity

The B16F10 cells were treated with α-MSH (100 nM) for 24 h, and then further treated with various concentrations of *A. brasiliensis* methanol extract (0.1, 0.25, 0.5 and 0.75 mg/mL) or arbutin (2.0 mM) for another 24 h. After these treatments, the cells were homogenized with 50 mM PBS (pH 7.5) buffer containing 1.0% Triton X-100 and 0.1 mM PMSF (phenylmethylsulfonyl fluoride; a serine proteinase inhibitor). The cellular extracts (100 µL) were mixed with freshly prepared L-DOPA solution (0.1% in phosphate-buffered saline) and incubated at 37°C for 30 min. The absorbance at 490 nm was measured with a microplate reader Gen 5™ (BIO-TEK Instrument, Winooski, VT., USA) to monitor the production of dopachrome.

Western blotting assay

The B16F10 cells treated with *A. brasiliensis* methanol extract (0.1, 0.25, 0.5 and 0.75 mg/mL) or Kojic acid (200 µM) were washed twice with cold PBS, lysed in PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 µg/mL of aprotinin, 100 µg/mL of phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL of pepstatin A, and 1 mM EDTA at 4°C for 20 min, and then the cells were disrupted with a needle. Proteins (50 µg) were resolved by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride (PVDF) filter. The nylon filter was blocked for 1 h in 5% fat-free milk in PBST buffer (PBS with 0. 05% Tween-20). After a brief wash in the PBST buffer, the nylon filter was incubated overnight at 4°C with the

following antibodies respectively: anti-tyrosinase antibody (1:2,000), anti-TRP1 antibody (1:2,000), anti-TRP2 antibody (1:2,000), anti-MITF antibody (1:1,000) and anti-GAPDH antibody (1:3,000) (Epitomics, Burlingame, CA). Then, the primary antibody was removed, and the filter was further washed extensively in PBST buffer. Subsequent incubation with goat anti-mouse antibody (1:10,000) conjugated with horseradish peroxidase was conducted at room temperature for 2 h. The filter was washed extensively in PBST buffer to remove the secondary antibody, and the blot was visualized with ECL reagent. The relative amounts of tyrosinase, TRP-1, TRP-2 and MITF were compared with total GAPDH and analyzed with Multi Gauge 3.0 software (Fuji, Tokyo).

Determination of intracellular ROS content

The B16F10 melanoma cells were cultured in 24-well plates (5×10^4 cells in 1 mL of DMEM medium) and treated with various concentrations of *A. brasiliensis* methanol extract (0.1, 0.25, 0.5 and 0.75 mg/mL), Trolox® (2.0 mM) or none for 24 h. The cells were then incubated with 24 mM H_2O_2 at 37°C for 30 min. After incubation, 2',7'-dichloro-fluorescein diacetate (DCFH-DA) was added to the wells, and the cells were cultured for 30 min. After treatment, the cells were washed with phosphate-buffered saline, and trypsinized with trypsin/EDTA. The fluorescence intensities of DCF were measured at an excitation wavelength of 504 nm and emission wavelength of 524 nm using a fluorescent reader, Fluoroskan Ascent (Thermo Scientific, Vantaa, Finland). The data were analyzed with Ascent software (Thermo Scientific, Vantaa, Finland). Cells with increased ROS level appeared as a population with high fluorescence intensity (Murrant and Reid, 2001).

Statistical analysis

Statistical analysis of the experimental data points was performed using the one-way ANOVA test, which compared measured data using SPSS 12.0 statistical software (SPSS INC. Chicago, IL, USA). The data are presented as the mean ± standard deviation of the triple experiments. Differences were considered as statistically significant when *p*-value was less than 0.05.

RESULTS AND DISCUSSION

Cell viability assay

The MTT assay results show in Figure 1 indicated that *A. brasiliensis* methanol extract shows no cytotoxicity to the B16F10 cells.

Determinations of mushroom tyrosinase activity, B16F10 cellular tyrosinase activity and melanin content

The inhibition percentage of mushroom tyrosinase activity was 19.41 ± 2.08, 33.59 ± 2.79 and 49.82 ± 0.51% of control for 1, 5 and 10 (mg/mL) of *A. brasiliensis* methanol extract, respectively. The IC_{50} of the methanol extract was 9.995 mg/mL. Moreover, the inhibition percentage for kojic acid (200 µM) was 48.95 ± 0.35% of the control (Figure 2A). Thus, *A. brasiliensis* methanol extract could act as a potential tyrosinase inhibitor. The melanin content in

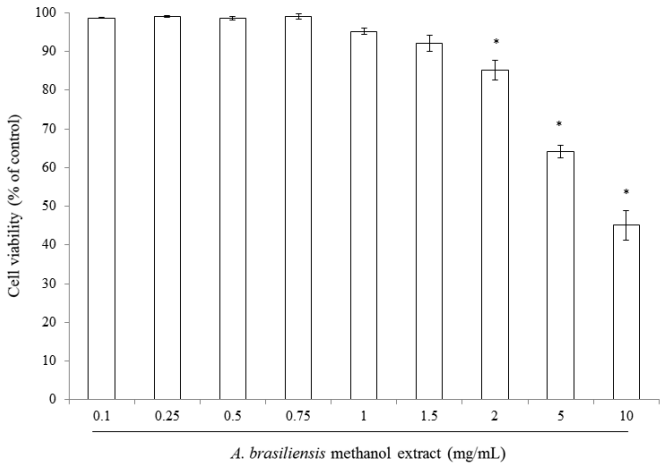

Figure 1. Cell viability assay. Results are expressed as the percentage of cell viability relative to the control. Data are presented as the mean ± S.D. Values werre significantly different by comparison with control. *$p < 0.05$.

B16F10 melanoma cells was 97.05 ± 3.52, 87.59 ± 1.88, 68.92 ± 1.34 and 45.57 ± 3.09% for 0.1, 0.25, 0.5 and 0.75 (mg/mL) of the *A. brasiliensis* methanol extract, respectively. The IC_{50} of the methanol extract was 0.713 mg/mL. The residual melanin content for arbutin was 66.15 ± 2.30% of control. The results show in Figure 2B indicated that the methanol extract from *A. brasiliensis* exhibit a strong inhibitory effect on melanin production. Recently, we have found that arbutin is more effective than kojic acid in the B16F10 cell model (Huang et al., 2012; Huang et al., 2012). Thus, we chose arbutin as the positive standard in the assays of melanin content and intracellular tyrosinase activity. The residual intracellular tyrosinase activity was 95.75 ± 0.84, 88.87 ± 1.86 , 67.12 ± 2.23 and 45.97 ± 1.84% for 0.1, 0.25, 0.5 and 0.75 (mg/mL) of the methanol extract, respectively. The IC_{50} of the methanol extract was 0.711 mg/mL. In addition, the intracellular tyrosinase activity was 66.53 ± 2.73% in the arbutin treated cells (Figure 2C). The results show in Figure 2C were in accordance with the results indicated in Figure 2B, which means that *A. brasiliensis*he methanol extract inhibited B16F10 intracellular tyrosinase activity and then decreased melanin production. In these experiments, α-MSH was used as a cAMP inducer to stimulate melanin synthesis. It is reported that α-MSH can bind the melanocortin 1 receptor (MC1R) and activate adenylate cyclase, which catalyzes ATP to cAMP and increases the intracellular cAMP level (Busca and Ballotti, 2000).

Determination of expression levels of melanogenesis-related proteins

The results show in Figure 3 reveal that *A. brasiliensis* methanol extract reduced protein expression levels of

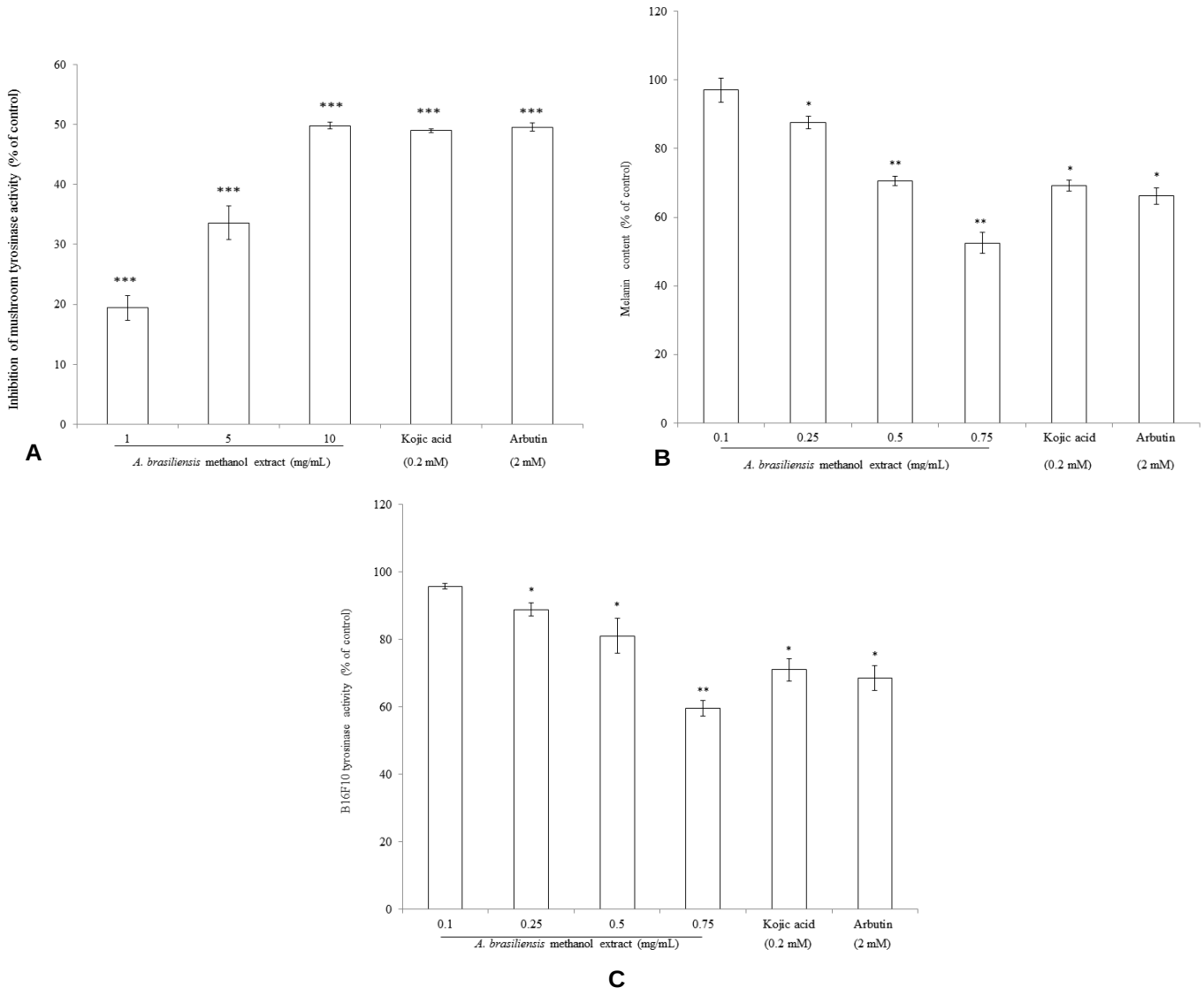

Figure 2. Inhibitory effect of *A. brasiliensis* methanol extract on melanogenesis. **A**. Mushroom tyrosinase activity assay. **B**. Determination of melanin content in B16F10 cells. **C**. Measurement of intracellular tyrosinase activity. The results are represented as percentages of the control. The data are presented as the mean ± SD for the three separate experiments. Values are significantly different by comparison with the control. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

TRP-1 and tyrosinase. It is reported that TRP-1 and TRP-2 regulate tyrosinase activity (Wan et al., 2011). The results indicated that *A. brasiliensis* methanol extract decreased the expression of TRP-1and tyrosinase (Figure 3B), which in turn inhibited tyrosinase activity and decreased melanin production in B16F10 cells.

Assays of ROS levels

The principle of the assay of intracellular ROS is that DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed to DCFH by esterase, then DCFH reacts with ROS (such as H_2O_2) to yield DCF. Rapid increases in DCF indicated oxidation of DCFH by intracellular radicals. The residual intracellular ROS induced by H_2O_2 (20 mM) was 88.87 ± 2.71, 74.94 ± 1.58, 53.62 ± 2.27, 30.75 ± 1.78% of control for 0.1, 0.25, 0.5 and 0.75 (mg/mL) of the methanol extract, respectively. The remaining intracellular ROS for 2.0 mM Trolox was 73.79 ± 2.58% of control (Figure 4). It has been reported that UV light irradiation induce the formation of ROS in cutaneous tissue provoking toxic changes such as lipid peroxidation and enzyme inactivation (Emerit, 1992). UV light irradiation plays a role in the initiation of several skin disorders, including scaling, wrinkling and hyperpigmentation Ichihashi et al., 2003). The results suggested that *A.* (*brasiliensis* methanol extract decreased melanin

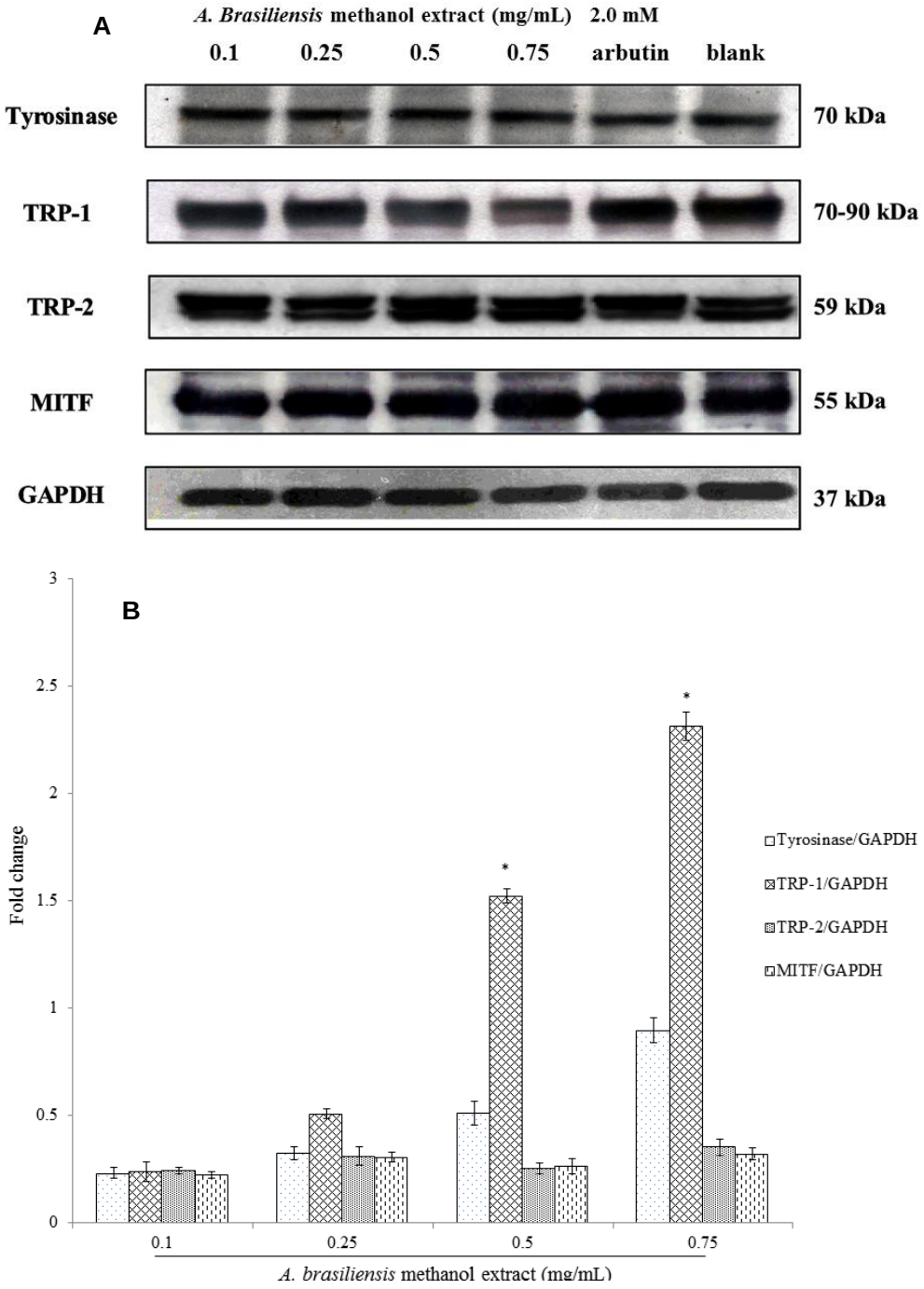

Figure 3. Western blotting analysis. **A**. Effect of *A. brasiliensis* methanol extract on the expression levels of the melanogenesis-related proteins. **B**. The relative amounts of tyrosinase and TRP-1 compared with the total GAPDH calculated and analyzed with the Multi Gauge 3.0 software. Results are represented as percentages of the control, and the data are presented as the mean ± SD for the three separate experiments. Values are significantly different compared with the control. *$p < 0.05$.

production may be attributed to its depletion of intracellular ROS.

In this study, *A. brasiliensis* methanol extract shows potent inhibitory effect on melanin production in B16F10 melanoma cells. Also, *A. brasiliensis* methanol extract decreased the expression levels of tyrosinase and TRP-1, depleted the cellular ROS content, then inhibited intracellular tyrosinase activity and decreased melanin

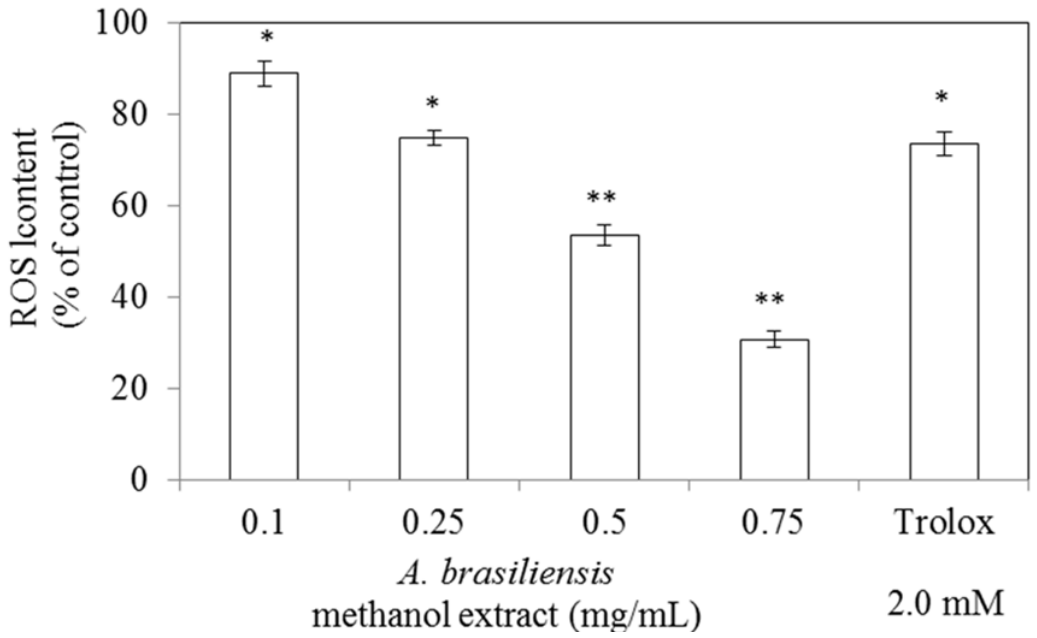

Figure 4. Effect of *A. brasiliensis* methanol extract on the intracellular ROS level. Results are expressed as the percentage relative to the control. Data are presented as the mean ± S.D. Values are significantly different compared with the control. *$p < 0.05$; **$p < 0.01$.

synthesis significantly. Thus, the *A. brasiliensis* methanol extract could thereby be used as a skin depigmenting agent in skin care products.

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