

Tyrosinase Activity and Melanogenic Effects of *Lespedeza bicolor* Extract *in vitro* and *in vivo*

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Lespedeza bicolor (*L. bicolor*) is used for medicinal purposes because of its various biological and pharmacological activities. In this study, the effects of *L. bicolor* ethanol extract on the treatment of vitiligo were investigated. The determination of melanin content in melanocytes was measured using B16 melanoma cells and C57BL/6J Ler-vit/vit mice. Finally, the quercetin content in *L. bicolor* were qualitatively analyzed using HPLC. The results obviously indicated that the *L. bicolor* extract enhanced melanogenesis and increased tyrosinase activity in cultured melanoma cells and C57BL/6J Ler-vit/vit mice. Treatment with *L. bicolor* extract led to a higher content of melanin and eumelanin in C57BL/6J Ler-vit/vit mice hair than in control (untreated) mice, which demonstrated the therapeutic effect of hair-graying associated with vitiligo. There was a notable increase in melanocytes in the skin of C57BL/6J Ler-vit/vit mice treated with *L. bicolor* extract compared with the control. *L. bicolor* extract was a potent tyrosinase and melanin synthesis activator in B16 melanoma cells. C57BL/6J Ler-vit/vit mice treated with *L. bicolor* extract had significantly higher melanin content in hair than the untreated control. The results suggest that *L. bicolor* extract is a potential alternative treatment for improvement of vitiligo.

Keywords: *Lespedeza bicolor*; Melanogenesis; Tyrosinase activity; Vitiligo

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INTRODUCTION

Vitiligo is usually an acquired pigment disorder, a skin disease caused by the loss of the melanin pigment in the epithelium. Vitiligo occurs in 1% of the world's population and affects all races. Different studies suggest that some genetic mechanisms are involved in the etiology of vitiligo; in some cases, at least 30% of people have a family history. The exact cause of this disease is not yet known, and only the period of remission and exacerbation is known. Depigmentation might be an option, but such radical transformations may have a serious impact on a patient's cultural identity.

In vitiliginous areas, melanocytes are present in the skin but are in an inhibited state of functional activity. When melanocyte activity is affected, melanin is not synthesized in the melanosomes of melanocytes. In addition, the development of vitiligo is related to the rates of synthesis and decay of tyrosinase. Many studies have investigated the importance of tyrosinase in the regulation of racial pigmentation. Song *et al.* (1994) showed that tyrosinase was an enzyme important in melanin formation.

The current treatment options for vitiligo include medication, surgery, and

adjunctive therapies (those used along with surgical or medical treatments). Whitton *et al.* (2016) reported that there are several ways to improve the appearance of vitiligo, but the effect is limited. Narrowband ultraviolet B (NB-UVB) phototherapy is problematic because the resulting repigmentation is transient. Skin grafting techniques are the most effective interventions, but they can only be used with stabilized or segmental types of vitiligo, which are less common. In summary, there is currently no satisfactory solution to vitiligo, and the patients are impacted for life.

The study of local knowledge of natural resources has become increasingly important in the investigation of the development of medicines without side effects. Many studies are underway to find potential pigment for the treatment of vitiligo. *Piper nigrum* L. fruit extract has growth-stimulatory activity in melanocytes. In addition, the extracts from *Cucumis melo* and *Ammi visnaga* fruits have been studied. Wood extracts have been tested also as a treatment for vitiligo. In studies by Szczurko *et al.* (2011), the potency of oral *Ginkgo biloba* extract to halt progression of active vitiligo was evaluated. The wood extract treatments studied were *Angelica sinensis* root extract and *Ammi majus* L. extract. Lin *et al.* (1999) found that out of 28 herbal extracts screened, significant stimulation ($p < 0.05$) of melanocyte proliferation was observed using aqueous extracts of herbs. Tahir *et al.* (2010) reported that *Polypodium leucotomos* extract has been used for the treatment of vitiligo for more than 10 years in Europe. Madhogaria and Ahmed (2010) reported a patient who developed depigmented patches after using a cream containing kojic dipalmitate, licorice root extract, and *Mitracarpus scaber* extract.

As a medicinal plant, *Lespedeza bicolor* (*L. bicolor*) is used in folk medicine for white patches on the body, such as leucoderma and vitiligo. Despite its established popular use for the treatment of vitiligo, there are few reports in the literature on this property. Similar previous studies have investigated the effects of flavonoids, contained in many *L. bicolor* extracts, on tyrosinase and the therapeutic effects on inflammation (Samiullah *et al.* 2012; Rafiq *et al.* 2013). In addition, quercetin, a component of *L. bicolor* extract, was reported to induce the upregulation of melanogenesis and enhance tyrosinase activity in dose- and time-dependent manners (Nagata *et al.* 2004).

Therefore, *L. bicolor* extract could be used as a potential resource for plant-based pharmaceutical products for melanogenesis. *In vitro* and *in vivo* experiments were conducted to determine the effect of *L. bicolor* extract on vitiligo treatment. The cytotoxicity, tyrosinase activity, and melanin content were analyzed in B16 melanoma cells *in vitro*. The melanin content, eumelanin content, and histologic analysis were examined in C57BL/6J Ler-vit/vit. A mice model for vitiligo, an acquired cutaneous depigmentary disorder, has been established and given the provisional genetic designation C57BL/6J Ler-vit/vit on Boissy *et al.* (1987). Through this study, the potential of *L. bicolor* for the treatment of vitiligo was examined.

EXPERIMENTAL

Materials

Preparation of extract

The plant materials were obtained from the Gyeongsang National University Research Forest, Jinju, South Korea and identified by comparison with the voucher specimen (HCCN: 24239 GBH0000003470) deposited at the Herbarium of the Rural Development Administration-Genebank Center, Jeonju, South Korea. The plants were

identified by Dr. Hak-gon Kim in the GyeongNam Forest Environmental Research Institute, Jinju, South Korea. The plants were authenticated by Hee-gon Kang in Gyeongsang National University Research Forest, Jinju, South Korea. The fresh stem of *L. bicolor* (Fig. 1) was cut into small pieces (length 3 cm) and dried over-night at room temperature. The dried, *L. bicolor* stem (500 g) was soaked in 60% ethanol 10 L for 1 week at room temperature. After vacuum filtration (Whatman No. 2 filter paper), the residue was extracted twice more in the same way. The filtrates were evaporated at 45 °C under reduced pressure using a rotary evaporator to remove the solvent and lyophilized to obtain the crude extract at a yield of 21.8% (109 g). The extract was stored at 4 °C.



Fig. 1. *Lespedeza bicolor*

Cell culture

Mouse melanoma cell lines were provided by Ajou University Medical Center. The B16 melanomas growth medium was composed of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA), 10% fetal bovin serum (FBS), and 1% penicillin/streptomycin (PS). The culture condition was 37 °C in a humidified atmosphere of 0.5% CO₂.

Cell Cytotoxicity Assay

Subcultures of B16 cells were seeded in 96 well plates at a density of 7×10^3 B16 cells and cultured for 24 h. The medium was then replaced with 500 μ L fresh DMEM medium containing 10% FBS and 1% PS. Next, 1 μ L (5, 10, 20 μ g *L. bicolor* ethanol crude extract/1 mL 60% ethanol) of the *L. bicolor* ethanol extract was added to each well and cultured for 3 day. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved in phosphate-buffered saline (PBS). After 3 days, 50 μ L of MTT (at a final concentration of 5 mg/mL) were added, and the cells were incubated in 0.5% CO₂ at 37 °C for 1 h. The plates were shaken with dimethylsulphoxide (DMSO) for 15 min to dissolve the blue/purple formazan crystals. The percentage of viable cells was quantified by identifying the ability to reduce MTT. The optical density was measured in a ELISA reader

(Tetertek Multiskan MCC/340, LabSystem, Helsinki, Finland) at 540 nm.

Determination of Melanin Content in Melanocytes

The assay followed Kubo's method (Kubo *et al.* 2004) with slight modification. Briefly, subcultures of B16 cells were seeded in 60 ϕ plates at a density of 8×10^4 B16 melanoma cell and cultured for 24 h. The medium was replaced with 3 mL fresh DMEM medium containing 10% FBS and 1% PS. The 1 μ L of the *L. bicolor* ethanol extract was added to each well and cultured for 3 day. After 3 days, the cells were harvested, suspended in 0.1 mL 1N NaOH-10% DMSO solution (v/v), and kept at 60 $^{\circ}$ C for 6 h in a water bath. The 90 μ L test solution was transferred into a 96 well plate and measured in a ELISA reader (Tetertek Multiskan MCC/340, LabSystem, Helsinki, Finland) at 490 nm. The melanin content was determined by calculation from a synthetic melanin standard curve.

Mushroom Tyrosinase Assay

Measurement of tyrosinase in L-DOPA oxidation of mushroom extracts was carried out as described by Masamoto *et al.* (2003). The experiment was performed by partially modifying the method. First, 100 μ L of 0.1 M phosphate buffer was mixed with 20 μ L of different concentrations from *L. bicolor* ethanol extract. Then, 20 μ L of mushroom tyrosinase (2,000 U/mL in phosphate buffer) were added to initiate the reaction. The mixture was incubated at 37 $^{\circ}$ C for 5 days and then incubated at 37 $^{\circ}$ C for 10 min with the addition of 40 microliters of L-DOPA (4 mM in 0.1 M phosphate buffer). The mixture was measured in a ELISA reader at 475 nm. The percentage activity of tyrosinase was calculated as follows,

$$\% \text{ activity} = 100 - (B/A \times 100) \quad (1)$$

where A is absorbance in 10 min without sample, and B is absorbance in 10 min with tested sample.

Determination of Cellular Tyrosinase Activity

Subcultures of B16 cells were seeded in 60 ϕ plates at a density of 8×10^4 B16 melanoma cell and cultured for 24 h. The medium was replaced with 3 mL fresh DMEM medium containing 10% FBS and 1% PS. The 1 μ L of the *L. bicolor* ethanol extract was added to each well and cultured for 3 days. After 3 days, the cells were harvested and lysed by incubation at -4 $^{\circ}$ C for 1 h in 100 μ L lysis buffer (PBS pH 6.8, trytone \times 100 1%). The lysates were centrifuged at 10,000 \times g for 30 min (4 $^{\circ}$ C) to obtain the supernatant as a source of tyrosinase. Tyrosinase activity was assayed as described previously (Nagata *et al.* 2004). The reaction mixture contained 20 μ g of lysate, 180 μ L of 2 mM L-DOPA/pH 6.8 PBS, and pH 6.8 PBS. After incubation at 37 $^{\circ}$ C for 1 h, Absorbance was measured at a wavelength of 490 nm to observe the dopachrome formation.

Determination of Melanogenesis in C57BL/6J Ler-vit/vit Mice

Cell culture

All the experimental procedures were performed according to the guidelines of the Committee for Ethical Usage of Experimental Animals at Gyeongsang National University, South Korea: 1.) Act on Experimental Animals Act No. 9932 2010.1.18, 2010.3.19 Other laws amended by the Ministry of Health and Welfare; 2.) Enforcement Decree of the Experimental Animal Act Presidential Decree No. 22075 2010.3.15, 2010.3.19 Other laws amended by the Ministry of Health and Welfare; 3.) Enforcement

Rules of the Act on Experimental Animals Ministry of Health and Welfare No. 1 2010.3.19, 2010.3.19 Other laws amended by the Ministry of Health and Welfare. The C57BL/6J Ler-vit/vit mice with melanocyte disappearance (Medrano and Nordlund 1990; Slominski and Paus 1993) were used as *in vivo* animal models. Female C57BL/6J Ler-vit/vit mice were purchased from SAMTAKO (Gyeonggi-do, South Korea). These mice were stored under the conditions of temperature (20 to 26 °C), humidity (30 to 70%), and illumination (lit from 08:00 to 20:00) and used for the experiment. The type of food for mice was standard diets, and crude nutrients were 20% protein, 4.5% fat, 6% fiber, 7% ash, 0.5% calcium, and 1% phosphorus. The bedding material was GLP bedding (SAMTAKO, Gyeonggi-do, South Korea), and number of cage companions was one. The C57BL/6J Ler-vit/vit mice used in this experiment were 10 to 15 weeks of age. Food and tap water were provided *ad libitum*. *L. bicolor* ethanol extract was dissolved in 60% ethanol and used in the experiment. The mice were randomly divided into two groups of five mice as normal control group with mice treated 60% ethanol and mice treated 0.2 mL/cm² *L. bicolor* ethanol extract for 5 months without intermission. The control was treated with 60% ethanol on the opposite side of the same mouse. Repeated experiments were conducted with three sets and changes in hair color was monitored on once a day. When the experiment finished, euthanization was performed by 10% isoflurane with prolonged exposure at 1, 2, and 4 h after administration, and death was confirmed by exsanguination.

Melanin and eumelanin content measurement in mice hair

Samples of hair were incubated overnight in 1M NaOH, as previously described (Green and Wilson 1996). Standards were prepared by dissolving synthetic melanin (Sigma Chemical Co., Poole, Dorset, UK) over the concentration range 0.05 to 0.4 mg/mL in 1 M NaOH. The absorbance at 500 nm (total melanin) and 650 nm (eumelanin) of both standards and sample digests was measured using a Pye Unicam SP8-100 ultraviolet-visible spectrophotometer. Because synthetic and endogenous melanin differ in structure, the values presented are comparative rather than absolute.

Histologic examination of C57BL/6J Ler-vit/vit mice

Throughout this investigation, the standard procedure of Laidlaw and Blackberg (1932) was used, and for carrying out the reaction both freshly prepared sheets of pure epidermis and frozen sections were employed. Where paraffin sections were found to be necessary, as when studying the distribution of melanocytes in hair follicles, specimens comprising the full thickness of the skin were subjected to Dopa treatment after a brief preliminary period of formol fixation. They were then given an additional period in the fixative and sectioned by ordinary methods. The dermo-epidermal was incubated with EDTA solution (pH 7.4) at 37 °C for 2 h. Dermo-epidermal was separated with microforcep and washing with saline for 1 min. The dermo-epidermal was incubated with the L-dopa solution at 37 °C for 1 h. The dopa solution after the reaction was replaced with a new dopa solution, and dermo-epidermal was once more incubated at 37 °C for 8 h.

Finally, dermo-epidermal was washed with saline for 1 min. Dyed dermo-epidermal was fixed with 10% formalin for 20 min and washed with distilled water for 3 min. Finally, the dermo-epidermal was dehydrated with 95% alcohol and 100% alcohol for 20 min each and cleared with the xylene for 20 min (three times). The dermo-epidermal was mounted on slide glass with Canada balsam.

HPLC Analysis

Chromatographic analysis was carried out by DAD following RP-HPLC separation installed with HIQ SIL C18V reversed-phase column (\varnothing 4.6 mm \times 250 mm, Young-In Chromass, Anyang-si, South Korea) packed with 5 μ m diameter particles. The mobile phase was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0 % acetic acid. The flow rate and injection volume were 1.0 mL/min and 10 μ L, respectively. The standard was quercetin, and both sample and standard were filtered through a 0.45 μ m membrane filter. HPLC analysis was performed at ambient temperature, and the peak analysis of the chromatography was confirmed by comparison with the retention time of the standard.

Statistical Analysis

All experiments were run at least in triplicate. SPSS 11.5 (Chicago, IL, USA) and PROC GLM in SAS 9.1 software (Cary, NC, USA) were used for the statistical analysis. A descriptive statistical analysis was made by calculating the mean and standard deviation, and comparison between groups was complemented by a comparison between means (pairwise t-test). $P < 0.05$ and $p < 0.001$ were interpreted as significant.

RESULTS AND DISCUSSION

Toxicity to B16 Melanoma Cells

The effects of the extract on cell proliferation were investigated. The cells were exposed to various doses of extract for 72 h, and cytotoxicity was determined by the MTT assay. As shown in Fig. 2, cell viability was maintained for 72 h after exposure to *L. bicolor* extract. The cells were treated with various concentrations of *L. bicolor* extract (5, 10, and 20 μ g/mL), and the cell viability was calculated relative to the control. Cell viability was maintained as *L. bicolor* extract concentration increased (5, 10, and 20 μ g/mL). These results indicated that *L. bicolor* extract effectively induced the survival of B16 cells. In addition, *L. bicolor* extract did not exert cytotoxic effects on B16 melanoma cell proliferation. The percentage viabilities of control cells and those treated with ethanol and various concentrations were not significantly different. Mosmann (1983) reported that the amount of formazan produced in the MTT assay is exactly proportional to the viability of the cells. Therefore, the effects of *L. bicolor* extract on melanin synthesis and cellular tyrosinase activity were examined subsequently.

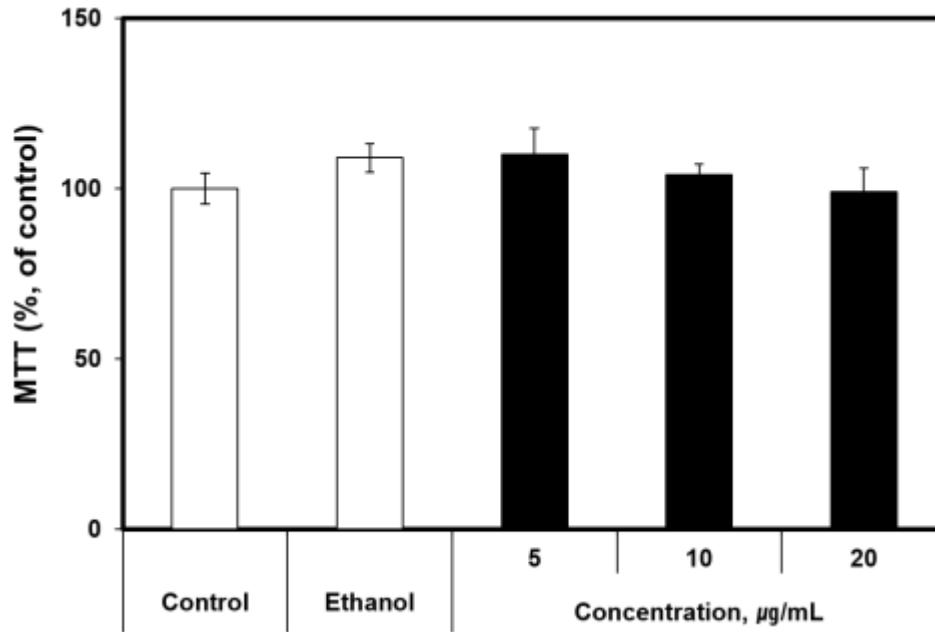


Fig. 2. Effect of *L. bicolor* ethanol extract on cytotoxicity in B16 melanoma cells. Control: untreated; Ethanol: 60 % ethanol; Concentration: *L. bicolor* ethanol extract

Effect of *L. bicolor* Extract on Melanin Content in B16 Melanoma Cells

B16 melanoma cells offer quantifiable markers of cytodifferentiation, such as melanin production, as well as a morphological marker (dendrite formation) (Pomerantz 1964). There is considerable experimental evidence to indicate that growth and melanization are intimately related in melanoma cells (Huberman and Callahan 1979). Lan *et al.* (2005) reported that melanin content was significantly higher in vitiligo lesions. Itoh and Furuichi (2005) used melanin content as an indicator for the evaluation of anti-graying effects in hair and improvements in vitiligo vulgaris. Niu *et al.* (2016) reported that the main cause of vitiligo was anti-melanogenic activity and that the analysis of melanin content was essential to improve vitiligo. To examine the melanogenic activity of the *L. bicolor* extract, the stimulatory effect of *L. bicolor* extract on melanin was evaluated in B16 melanoma cells. The B16 melanoma cells were treated with the *L. bicolor* extract at 5, 10, and 20 µg/mL for 72 h. The melanin content was presented as a percentage of the control (vehicle). The following effects of *L. bicolor* extract on melanogenesis of the B16 melanoma cells were noted. *L. bicolor* ethanol extract exerted a marked stimulatory effect on melanogenesis, without affecting cell proliferation, at concentrations of 5 to 20 µg/mL (Fig. 3).

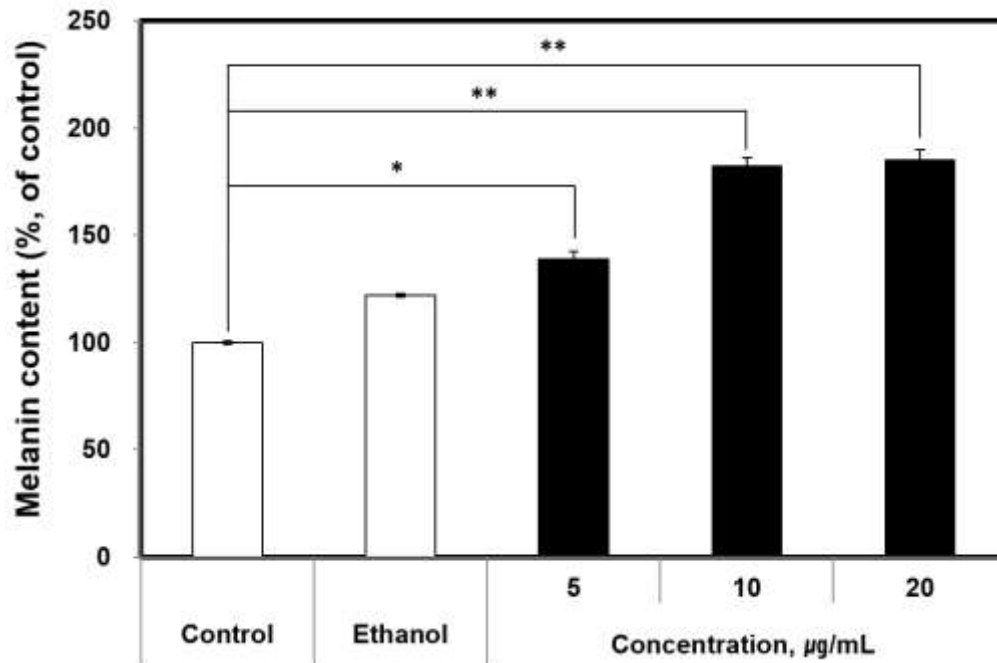


Fig. 3. Effect of *L. bicolor* ethanol extract on melanin content in B16 melanoma cells. Control: untreated; Ethanol: 60 % ethanol; Concentration: *L. bicolor* ethanol extract. * $p < 0.05$ compared to the untreated control; ** $p < 0.01$ compared to the untreated control.

The *L. bicolor* extract exhibited a significant dose-dependent increase on melanin content. The melanin content was $142.11\% \pm 0.07\%$, $180.00\% \pm 0.10\%$, and $181.95\% \pm 0.38\%$ after treatment with 5, 10, and 20 $\mu\text{g/mL}$ *L. bicolor* extract, respectively. Kang *et al.* (2018) reported that the melanin content was 146% and 110% after treatment with 8 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$ of *Euphorbia supina* extract in B16F10 cells, respectively. After the addition of 0.5 mM glycyrrhizin, the cellular melanin content reaches approximately 160% of control cells (Jung *et al.* 2001). The Tunisian *Capparis spinosa* extract stimulates melanogenesis in B16 cells and has been found to increase melanin content by 12% and 60% at 0.005% and 0.05% extract concentrations, respectively (Matsuyama *et al.* 2009). The *L. bicolor* extracts induced similar levels of melanin as previous studies, with a maximum melanin content of 181.95% after treatment with 20 $\mu\text{g/mL}$ extract. Therefore, the *L. bicolor* extract has the potential as a new natural resource to increase melanin content.

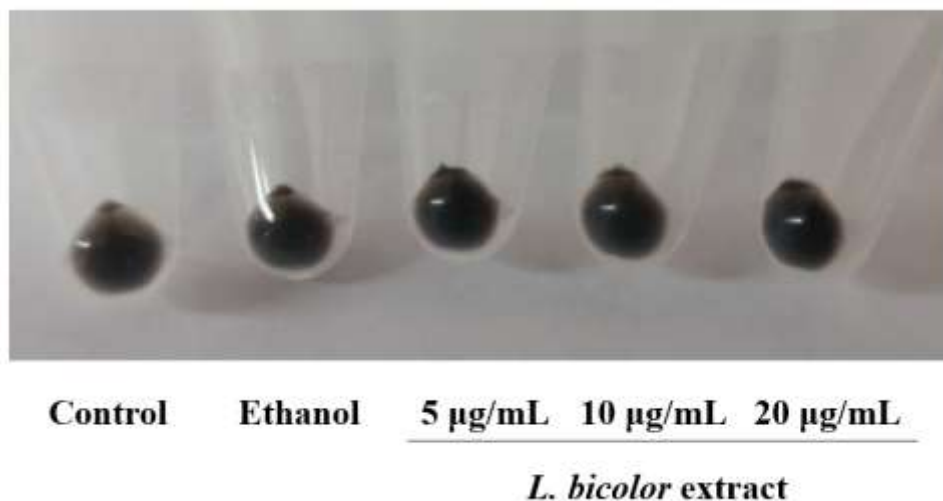


Fig. 4. *L. bicolor* extract increases melanogenesis in B16 melanoma cells. The dark-black color of the B16 melanoma cell pellets demonstrate that control (untreated), ethanol and the *L. bicolor* extract. Control: untreated; Ethanol: 60 % ethanol

In confirmation of the results of Fig. 4, the increase of melanin biosynthesis and secretion shows a distinct black color of the cell pellet. Hamid *et al.* (2012) reported that the dark-black color of the B16F1 melanoma cell pellets demonstrated that α -MSH, forskolin, and mangosteen leaf extract stimulated melanogenesis activity. Nair *et al.* (2001) evaluated repigmentation, as determined by cell pellet color and melanin assays. The colors of cell pellets are evaluated visually for melanin content assay (Usuki *et al.* 2003). These observations suggest that the *L. bicolor* extract increased the melanogenic activity of B16 melanoma cells.

Effect of *L. bicolor* Extract on Tyrosinase Activity

Effect of L. bicolor extract on mushroom tyrosinase activity

To determine whether *L. bicolor* ethanol extract had a direct effect on the major enzyme in the melanogenesis, an *in vitro* cell-free mushroom tyrosinase assay was conducted. The effects of *L. bicolor* ethanol extract on mushroom tyrosinase activity are shown in Fig. 5. The effects of the extract on the oxidation of L-DOPA by mushroom tyrosinase occurred in a dose-dependent manner. At 10 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$, *L. bicolor* ethanol extract exerted activity on the oxidation of L-DOPA by mushroom tyrosinase; the 10 $\mu\text{g/mL}$ *L. bicolor* ethanol extract showed lower activity than the 20 $\mu\text{g/mL}$ *L. bicolor* ethanol extract. However, there was no significant difference between the two concentrations. Tyrosinase catalyzes 3,4-dihydroxyphenylalanine (DOPA) quinone formation from DOPA, and melanin formation from DOPA quinone *via* autoxidation and enzymatic reaction (Jimenez *et al.* 1993). Therefore, melanin production is related to tyrosinase expression; the results presented here were similar. More specifically, both the melanin content and tyrosinase activity were increased by 10 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$ *L. bicolor* extract (Figs. 3 and 5). It is anticipated that continued research will increase knowledge concerning the activity of tyrosinase in *L. bicolor* ethanol extract, and continue to shed light on therapeutic strategies that can be used to reduce or eliminate vitiligo. Most studies have been performed on the effect of the mushroom tyrosinase from various plant extracts (Kim *et al.* 2003; Hsu *et al.* 2007). Thus, *L. bicolor* extract may be noted as an effective material with mushroom tyrosinase activity.

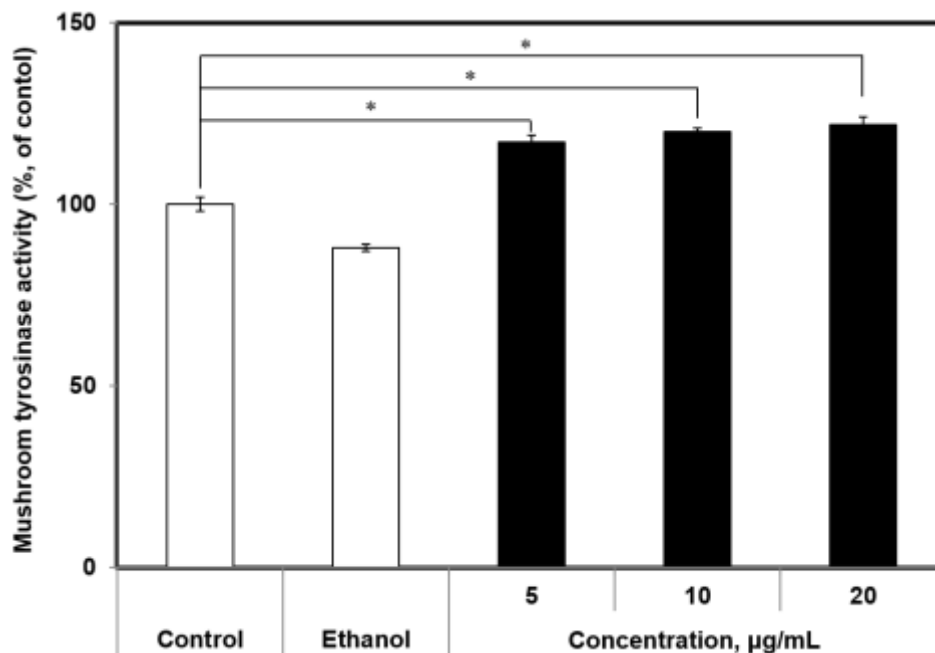


Fig. 5. Effects of *L. bicolor* ethanol extract on mushroom tyrosinase activity. Purified tyrosinase was mixed with *L. bicolor* ethanol extract and incubated with L-DOPA. Control: untreated; Ethanol: 60 % ethanol; Concentration: *L. bicolor* ethanol extract. * $p < 0.05$ compared to the untreated control.

Effect of L. bicolor extract on tyrosinase activity in B16 melanoma cells

Tyrosinase catalyzes three steps in the biosynthesis process of melanin; hence, the measurement of tyrosinase activity is very important. The intracellular tyrosinase activity was measured after the culture of B16 melanoma cells with *L. bicolor* ethanol extract. Different concentrations of *L. bicolor* extract, not mushroom tyrosinase, were used to treat cell lysats extracted from B 16 melanoma cells. Equal masses of cell lysate were prepared with respect to the protein concentration. At 5 µg/mL *L. bicolor* extract, a weak effect on the direct activation of intracellular tyrosinase was observed, but tyrosinase-inducing activity was increased significantly by 10 µg/mL and 20 µg/mL *L. bicolor* extract (Fig 6). Chen *et al.* (2012) showed that tyrosinase was regarded as the rate-limiting enzyme of melanogenesis, which modulates this process through the catalysis of the hydroxylation of tyrosine into DOPA and the further oxidation of DOPA into dopaquinone. Matsuda *et al.* (2005) confirmed the potential stimulation of melanogenesis from tyrosinase activity by using cultured B16 melanoma cells. The activity of tyrosinase in melanocytes may be expressed in tyrosinase cells due to direct activity or an increase in the total amount of protein in the cell (Oh *et al.* 2011). In the previous results, *L. bicolor* extract exerted a significant influence on tyrosinase activity. This result shows that the *L. bicolor* extract can be used directly as a tyrosinase activator. In a previous study (Jung *et al.* 2001), the cellular tyrosinase activity was also increased dose-dependently by glycyrrhizin, reaching 220% of the value in control cells at a treatment concentration of 1 mM. Adzuki bean extract is known to have a weak effect on the direct activation of tyrosinase in cells (Itoh and Furuichi 2005). Tuerxuntayi *et al.* (2014) reported that, compared with untreated conditions, treatment with Kaliziri extract at 5 to 40 µg/mL resulted in a dose-dependent increase in tyrosinase activity in B16 cells (to a maximum of 138% tyrosinase activity). Treatment with forskolin significantly increased the intracellular tyrosinase activity by more than

three-fold, whereas treatment with 32 $\mu\text{g/mL}$ extract demonstrated a four-fold increase in intracellular tyrosinase activity (Hamid *et al.* 2012). These results suggested that *L. bicolor* extract is a stimulant of tyrosinase, similar to other natural extracts; moreover, melanin synthesis increases as tyrosinase activity increased.

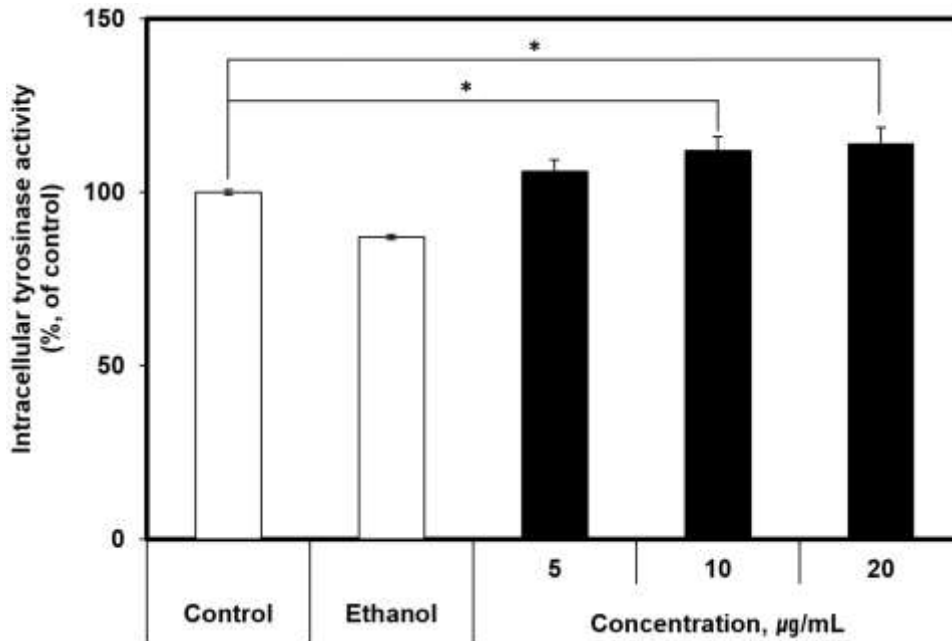


Fig. 6. Effect of *L. bicolor* ethanol extract on intracellular tyrosinase activity in B16 melanoma cells (Control: untreated; Ethanol: 60% ethanol treated).

* $p < 0.05$ compared to the untreated control.

Effect of *L. bicolor* Extract on Melanin Content in C57BL/6J Ler-vit/vit Mice Hair

Before the experiment, the health status of the mice were normal. The experiment was conducted using a total of 15 mice, which were used for all experiments. Melanin contents in the hair samples taken from the back of C57BL/6J Ler-vit/vit mice were significantly higher in those treated with *L. bicolor* ethanol extract than in the control group ($p < 0.01$) (Fig. 7). Therefore, the *L. bicolor* extract has the potential to assist repigmentation in vitiligo because it increased melanin content by approximately 40% compared with the control. In vertebrates and higher mammals, melanin plays an important role in thermoregulation, gastrointestinal tract, sexual attraction, and photoprotection. These melanin pigments can be distinguished chemically by the red-yellow pheomelanin and the brown-black eumelanin. Both types of melanin are found in human hair, the epidermis, and cultured melanocytes. The main synthesis process for these two pigments is similar and is controlled by tyrosinase. However, the production of eumelanin is important for repigmentation in the treatment of vitiligo.

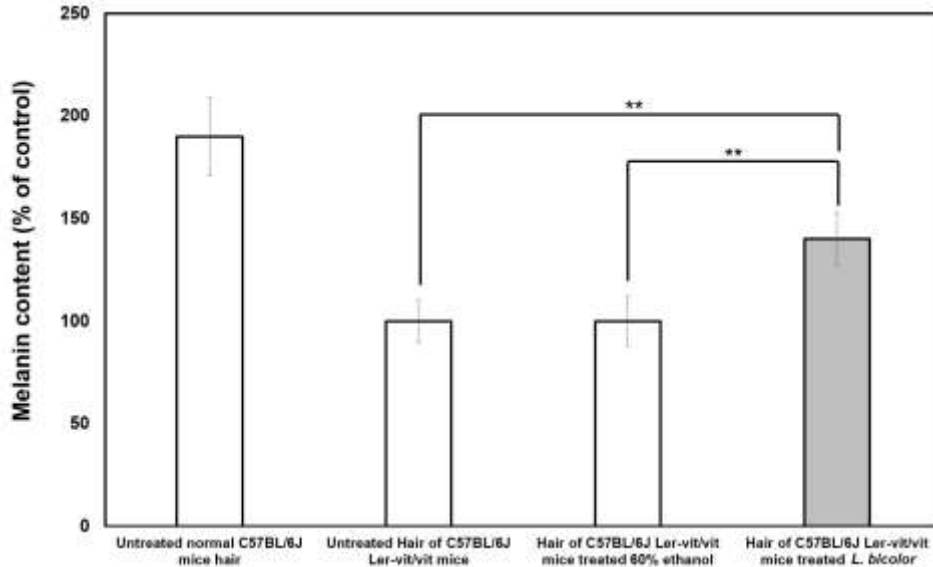


Fig. 7. Effect of *L. bicolor* ethanol extract on melanin content of hair in C57BL/6J Ler-vit/vit mice. The mice hair was lysed with 1N NaOH and the absorbance of the solution at 500nm was measured. C57BL/6J Ler-vit/vit mice: vitiligo mice hair; C57BL/6J: normal mice hair. ** $p < 0.01$ compared to the control

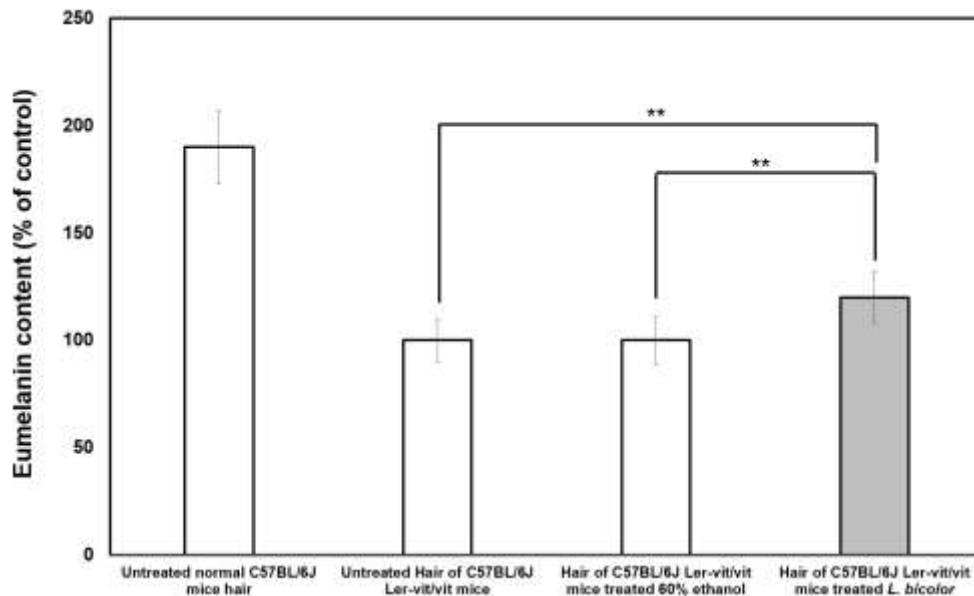


Fig. 8. Effect of *L. bicolor* ethanol extract on eumelanin content of hair in C57BL/6J Ler-vit/vit mice. The mice hair was lysed with 1N NaOH and the absorbance of the solution at 650 nm was measured. C57BL/6J Ler-vit/vit mice: vitiligo mice hair; C57BL/6J: normal mice hair. ** $p < 0.01$ compared to the control

The effect of *L. bicolor* extract on the eumelanin content of mouse hair is shown in Fig. 8. Treatment with *L. bicolor* extract resulted in significantly higher eumelanin content than the control (60% ethanol) in the hair of C57BL/6J Ler-vit/vit mice hair ($p < 0.01$). Although treatment with *L. bicolor* extract resulted in a lower eumelanin content than the hair of the normal C57BL/6J mice, the *L. bicolor* extract was effective in increasing the eumelanin content in mice hair. Yonemoto *et al.* (1983) reported a decrease in eumelanin

content in the lesions of vitiligo induced by 4-tertiary butyl catechol. Vitiligo occurs when there is a decrease in melanocytes and, in particular, when eumelanin is not formed (Prasad *et al.* 2003). Vitiligo affects the skin, head, and other parts of the body (Mihăilă *et al.* 2019). Thus, the results suggest that *L. bicolor* extracts can be used for repigmentation in the treatment of vitiligo.

Effect of *L. bicolor* extract on Melanin Cells in the Histology of C57BL/6J Ler-vit/vit Mice

Melanin cells around hair follicles observed under an optical microscope correspond, by their localization, to the DOPA-positive cells. Their morphological features are characteristic of melanocytes. Figure 9 shows representative sections of C57BL/6J Ler-vit/vit mice skin stained for melanin. Microscopic examination revealed pigmented areas, with more repigmentation after treatment with *L. bicolor* ethanol extract than the 60% ethanol used as a control. *L. bicolor* extract promoted a melanocytic response and the concentration required for the promotion of pigmentation was 0.2 mL/cm². These observations confirmed that *L. bicolor* extract restored vitiligo pigmentation. Interestingly, *L. bicolor* extracts cause a lot of pigmentation around mice hair follicles. The presence of melanocytes in the surrounding the root of mice hair follicles has already been documented (Ito and Wakamatsu 2003). This was consistent with the scientific literature affirming that repigmentation occurs mainly in the areas of skin where there were still pigmented hairs, as this suggests the presence of melanin reservoirs (Menon *et al.* 2016). Thus, the repigmentation of the epidermis following dermabrasion originates from the melanotic portion of the hair follicle. These results confirmed that *L. bicolor* extract was an effective stimulator of the melanin stored around the hair follicles.

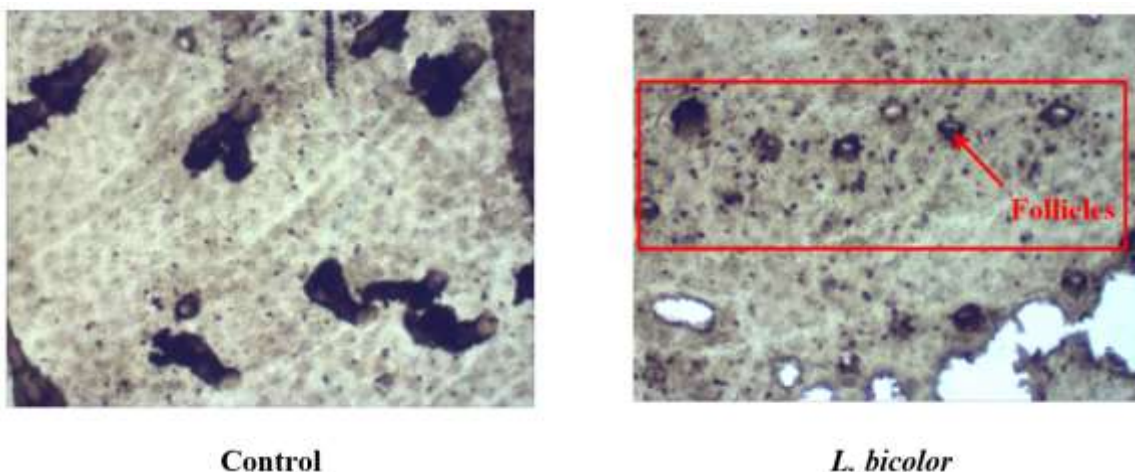


Fig. 9. Histologic examination of C57BL/6J Ler-414 vit/vit mice skin sections stained for melanin ($\times 100$). Control: C57BL/6J Ler-vit/vit mice treated 60% ethanol; *L. bicolor*: C57BL/6J Ler-vit/vit mice treated *L. bicolor* ethanol extract applied 0.2 mL/cm²

High-performance Liquid Chromatography (HPLC) Detection of Quercetin in *L. bicolor* Extract

L. bicolor extract was reported to contain flavonoid compounds, such as quercetin, kaempferol, trifolin, isoquercitrin, and homoorietin (Qi *et al.* 2011). A quercetin was previously isolated as the principal regulator of tyrosinase from the dried flower of *Heterotheca inuloides* Cass (Compositae), known as “arnica” in Mexico (Rodríguez-

Chávez *et al.* 2015). In addition, quercetin was previously reported to control the oxidation of 1-3,4-dihydroxyphenylalanine (L-DOPA, 2) catalyzed by mushroom tyrosinase (Kubo *et al.* 1994). Studies have investigated the effects of quercetin on tyrosinase activity and melanogenesis (Takekoshi *et al.* 2013). Nagata *et al.* (2004) demonstrated that the treatment of cultured melanoma cells with quercetin enhanced melanogenesis and also increased tyrosinase activity. HPLC analysis was performed to determine if quercetin was contained in the *L. bicolor* extracts that were produced by the authors. The application of HPLC to the study of quercetin in *L. bicolor* extract is shown in Fig. 10. The *L. bicolor* extract had the same retention time as the quercetin standard, which meant that quercetin was present in the *L. bicolor* extract. This was similar to previous studies that reported quercetin was contained in *L. bicolor* extracts (Glyzin *et al.* 1970). Therefore, it appears that quercetin plays a role in the melanogenic activity of *L. bicolor* extracts. Moreover, *L. bicolor* extract is a potential source of quercetin for melanogenesis.

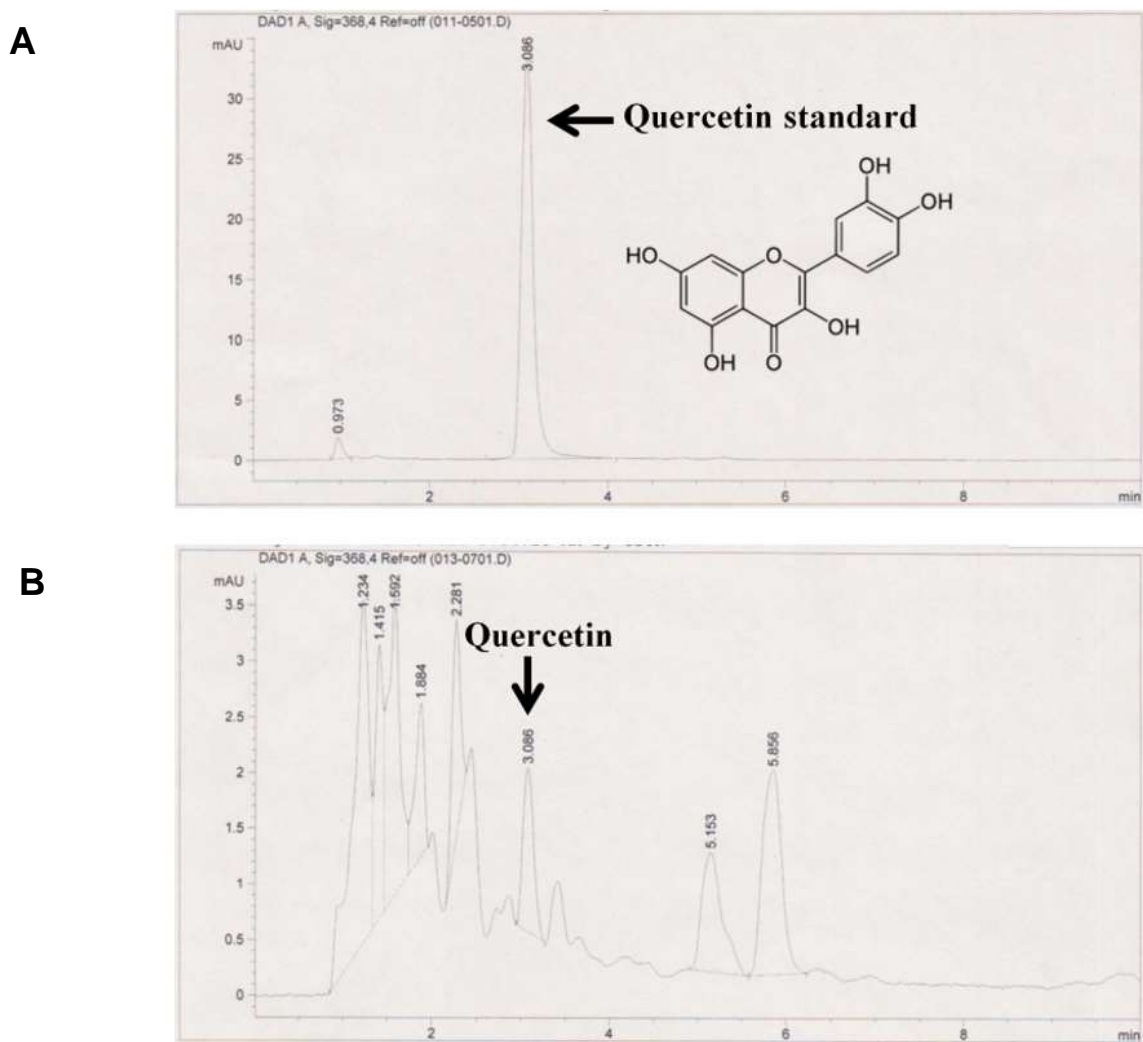


Fig. 10. The content of quercetin of the standard and *L. bicolor* extract by HPLC were shown. A: Quercetin standard (Retention time: 3.086 min), B: *L. bicolor* extract.

CONCLUSIONS

1. The effects of extract from *L. bicolor* on melanogenesis and tyrosinase activity were evaluated in B16 melanoma cells and C57BL/6J Ler-vit/vit mice. The results demonstrated the *L. bicolor* extract enhanced melanogenesis and increased tyrosinase activity in cultured melanoma cells and C57BL/6J Ler-vit/vit mice.
2. Treatment with *L. bicolor* extract led to a higher content of melanin and eumelanin in C57BL/6J Ler-vit/vit mice hair than in control (60% ethanol) mice, which demonstrated the therapeutic effect of hair-graying associated with vitiligo.
3. There was a notable increase in melanocytes in the skin of C57BL/6J Ler-vit/vit mice treated with *L. bicolor* extract compared with the control.
4. This study provides experimental evidence that *L. bicolor* could be used as an effective treatment for the treatment of vitiligo and other skin diseases.

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