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Original Article

Tyrosinase Activity and Melanogenic Effects of *Rhododendron schlippenbachii* Extract *In vivo* and *In vitro*[1]

Si Young HA[2] · Ji Young JUNG[2] · Hee Young KANG[3] · Tae-Heung KIM[4] · Jae-Kyung YANG[2,†]

ABSTRACT

Rhododendron schlippenbachii have been used as a medicine because of their various biological activities. In this study, *R. schlippenbachii* ethanol extract was evaluated for the treatment of vitiligo. The *R. schlippenbachii* ethanol extract did not show any cell cytotoxicity. The effect on mushroom tyrosinase and cellular tyrosinase activities were further assessed. In addition, the determination of melanin content in melanocytes was measured using both the B16 melanoma cells and C57BL/6J Ler-*vit*/*vit* mice. Finally, the existence of quercetin in *R. schlippenbachii* was confirmed by qualitative analysis using HPLC. The results clearly demonstrated the *R. schlippenbachii* extract enhanced melanogenesis and also increased tyrosinase activity in cultured melanoma cells and C57BL/6J Ler-*vit*/*vit* mice. In addition, treatment with *R. schlippenbachii* 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. Finally, we confirmed a notable increase in melanocytes in the skin of C57BL/6J Ler-*vit*/*vit* mice treated with *R. schlippenbachii* extract compared with the control. Extracts of *R. schlippenbachii* was shown to be potent tyrosinase and melanin synthesis activator in B16 melanoma cells. The *R. schlippenbachii* extract have significantly higher melanin content than the untreated control in C57BL/6J Ler-*vit*/*vit* mice hair. The results suggest that *R. schlippenbachii* extract might be considered as an alternative treatment for improvement of vitiligo.

Keywords: *Rhododendron schlippenbachii*, tyrosinase activity, melanogenesis, vitiligo

1. INTRODUCTION

Vitiligo is a common depigmenting skin disease, associated with certain autoimmune endocrinopathies, and autoantibodies to several antigens can be found in melanoma cells. Vitiligo is an acquired condition without other apparent presymptoms in the skin (Spritz, 2010). It was suggested that there were melanocytes but their melanin producing activity was inhibited. 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. As present, 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

[1] Date Received November 5, 2019, Date Accepted February 10, 2020
[2] Division of Environmental Forest Science/Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea
[3] Department of Dermatology, Ajou University School of Medicine, Suwon 16499, Republic of Korea
[4] White-Line Skin Clinic, Changwon 51496, Republic of Korea
† Corresponding author: Jae-Kyung YANG (e-mail: jkyang68@gmail.com, ORCID: 0000-0003-0423-6398)

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 known to be 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. Therefore, the study of local knowledge of natural resources has become increasingly important in the investigation of the development of medicines without side effects (Min *et al*., 2017; Manurung *et al*., 2019; Chen *et al*., 2018). Many studies are underway to find potential pigment for the treatment of vitiligo and, *Piper nigrum L*. fruit extract has been shown to have growth-stimulatory activity in melanocytes (Lin *et al*., 1999). In addition, the natural resource extracts were studied Cucumis melo extract (Schallreuter *et al*., 2011) and Ammi visnaga fruits (Sidi and Bourgeois-Gavardin, 1952). Wood extracts were also tested as a treatment for vitiligo. In the pilot study of Szczurko *et al*. (2011) and Abu-Raghif *et al*. (2013), the potency of oral *Ginkgo biloba* extract to halt progression of active vitiligo was evaluated. 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 *polylpodium 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. *Rhododendron schlippenbachii* (*R. schlippenbachii*) is widely distributed in Kurram Agency, Pakistan and the adjoining area in Afghanistan from 2000~3000 m. Some species of the genus *R. schlippenbachii* have been used as medicinal plants. The dried of *R. schlippenbachii* are used medicinally as an expectorant and treatment of acute-chromic bronchitis (Zhou *et al*., 1997).

From then *R schlippenbachii*, systematic and comprehensive investigations of the genus *Rhododendron* were performed worldwide, from which hundreds of secondary metabolites have been isolated, mainly flavonoids and diterpenoids. Some of the isolates show various kinds of significant bioactivities (Chen *et al*., 2008). In addition, quercetin, a component of *R. schlippenbachii* extract, was reported to induce the upregulation of melanogenesis and enhance tyrosinase activity in dose- and time-dependent manners (Nagata *et al*., 2004). Therefore, *R. schlippenbachii* 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 *R. schlippenbachii* 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, we aimed to confirm the potential of *R. schlippenbachii* for the treatment of vitiligo.

2. MATERIALS and METHODS

2.1. Preparation of extract

The plant materials were obtained from the experiment forest of Gyeongsang National University, Jinju, South Korea. The fresh stem of *R. schlippenbachii* was cut into small pieces (about length 3 cm) and dried overnight at room temperature. The dried, *R. schlippenbachii* stem (500 g) were soaked in 98% ethanol 10 L for 1 week

at room temperature. After vacuum filtration (Whatman No. 2 filter paper, ADVANTEC), the residue was extracted twice more in the same way as above. 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 before further processing.

2.2. Cell culture

B16 melanomas cell lines were provided by the dermatology of Ajou University Medical Center (164, World Cup-ro, Yeongtong-gu, Suwon, South Korea). 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 humidif ied atmosphere with 0.5% CO_2.

2.3. 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 hours. The medium was then replaced with 500 μ L fresh DMEM medium containing 10% FBS and 1% PS. 1μ L(5, 10, 20μ g *R. schlippenbachii* ethanol crude extract /1 mL 60% ethanol) of the *R. schlippenbachii* ethanol extract were added to each well and cultured for 3 days. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved in phosphate-bufferedsaline (PBS). After 3 days culturing, aliquots of MTT (at a final concentration of 5 mg/mL) were added 50 μ L and then the cells were incubated in a 0.5% CO_2 incubator at 37℃ for 1 hour. The plates were then shaken with dimethylsulfoxide (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 an ELISA reader (Tetertek Multiskan MCC/340, Labsystem, Helsinki, Finland) at 540nm (Ahn *et al*., 2018).

2.4. 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 ø plates at a density of 8×10^4 B16 melanoma cell and cultured for 24 hours. The medium was then replaced with 3 mL fresh DMEM medium containing 10% FBS and 1% PS. The 1μ L of the *R. schlippenbachii* ethanol extract were added to each well and cultured for 3 days. After 3 days culturing, the cells were harvested and suspended in 0.1 mL 1N NaOH-10% DMSO solution (v/v), kept at 60℃ for 6 hours in water bath. The 90 μ L test solution was transferred into 96 well plate and measured in an ELISA reader at 490nm. The melanin content was determined by calculation from a synthetic melanin standard curve.

2.5. Mushroom tyrosinase assay

Measurement of tyrosinase in L-DOPA oxidation of mushroom extracts was carried out quoted Masamoto *et al*. (2003). The experiment was performed by partially modifying the method. A 100 μ L of 0.1 M phosphate buffer was mixed with 20 μ L of different concentrations from *R. schlippenbachii* 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℃ for 5 days and then incubated at 37℃ for 10 min with the addition of 40 μ L of L-DOPA (4 mM in 0.1 M phosphate buffer). The mixture was measured in an ELISA reader at 475 nm. The percentage activity of tyrosinase was calculated as follows: % activity = 100 - (B/A x 100), where A = ΔOD_{475} in 10 min without sample, and B= ΔOD_{475} in 10 min with tested sample.

2.6. Determination of cellular tyrosinase activity

Subcultures of B16 cells were seeded in 60 ø plates at a density of 8×10^4 B16 melanoma cell and cultured for 24 hours. The medium was then replaced with 3 mL fresh DMEM medium containing 10% FBS and 1% PS. The 1㎕ L of the *R. schlippenbachii* ethanol extract were added to each well and cultured for 3 days. After 3 days culturing, the cells were harvested and lysed by incubation at −4℃ for 1 hour in 100 ㎕ L lysis buffer (PBS pH 6.8, 1% trytone×100). The lysates were centrifuged at 10,000× g for 30 min (4℃) to obtain the supernatant as a source of tyrosinase. Tyrosinase activity was assayed as described previously (Masamoto *et al*., 2003). The reaction mixture contained 20 ㎕ g lysate, 180 ㎕ L 2 mM L-DOPA/pH 6.8 PBS and pH 6.8 PBS. After incubation in the presence at 37℃ for 1 hour, Absorbance was measured at a wavelength of 490 nm to observe the dopachrome formation.

2.7. Application of the extract on C57BL/6J Ler−*vit*/*vit* mice skin

All the experimental procedures were performed according to the guidelines of the Committee for Ethical Usage of Experimental Animals at Gyeongsang National University. 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 (20-26℃), humidity (30-70%) 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 this experiment were 10-15 weeks of age. Food and tap water were provided *ad libitum*. *R. schlippenbachii* ethanol extract was dissolved in 60% ethanol and used in the experiment. The mice were randomly divided into two groups of five mice as group with mice treated 60% ethanol and mice treated 0.2 mL/cm^2*R. schlippenbachii* ethanol extract for 5 months without intermission. The control was treated 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 experiment finish, euthanization was performed by 10% isoflurane with prolonged exposure at 1, 2, and 4 h after administration, and death was confirmed by exsanguination.

2.8. 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, U.K.) over the concentration range 0.05~0.4 mg/mL in 1M 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. It should be noted that synthetic and endogenous melanin differ in structure, and hence, values presented are comparative rather than absolute.

2.9. 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. The 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. Incubation the split with EDTA solution (pH 7.4) was carried out at 37℃ for 2 hours. Dermo-epidermal was separated with microforcep and washing with saline for 1 min. And than, the dermo-epidermal was incubated with the L-dopa solution at 37 ℃ for 1 hour. After change the final L-dopa solution, dermo-epidermal was once more incubation at 37 ℃ for 8 hours and washing with saline for 1 min. Dyed dermo-epidermal was fixation with 10% formalin for 20 min and washing with distilled water for 3 min. Finally, dermo-epidermal was dehydration with 95% alcohol and 100% alcohol one by one for 20 min and clearing with the xylene for 20 min (three times). Dermo-epidermal on slide glass, it was mounting with canada balsam.

2.10. HPLC analysis

Chromatographic analysis was carried out by DAD following RP-HPLC separation installed with HIQ SIL C18V reversed-phase column (ø 4.6 mm × 250 mm) packed with 5μ m diameter particles, the mobile phase was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0 % acetic acid. Flow rate and injection volume were 1.0 mL/min and 10 μ L, respectively. The sample 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 cordycepin standard.

2.11. Statistical analysis

All the experiments were run at least in triplicate. SPSS 11.5 (SPSS Inc. Chicago) and PROC GLM in SAS 9.1 software (SASs Inc., Cary, NC) were used for all 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 $p < 0.001$ are interpreted as significant.

3. Results and Discussion

3.1. Toxicity to B16 melanoma cells

To evaluate the effects of the extract on cell proliferation, we investigated the effects of the extract on cell growth. The cells were exposed to various doses of extract for 72 h and cytotoxicity was determined by the MTT assay. As shown in Fig. 1, cell viability was maintained for 72 h after exposure to *R. schlippenbachii* extract. The cells were treated with various concentrations of *R. schlippenbachii* extract (5, 10, and 20 μg/mL) and the cell viability was calculated relative to the control. Cell viability was maintained as *R. schlippenbachii* extract concentration increased. These results indicated that *R. schlippenbachii* extract effectively induced the survival of B16 cells. In addition, *R. schlippenbachii* extract did not exert cytotoxic effects on B16 melanoma cell proliferation. The cell viability of control, treated with 60% ethanol and treated various

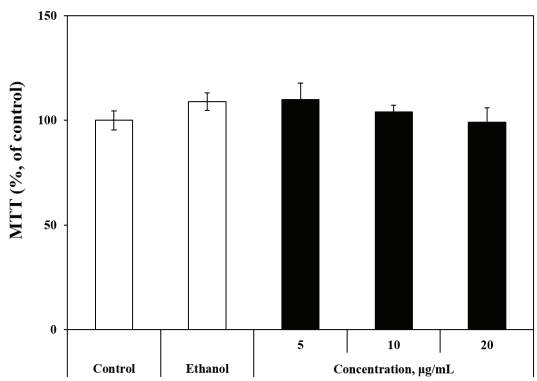


Fig. 1. Effect of *R. schlippenbachii* ethanol extract on cytotoxicity in B16 melanoma cells. Control: untreated; Ethanol: 60 % ethanol; Concentration: *R. schlippenbachii* ethanol extract.

concentration of *R. schlippenbachii* extract 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, we subsequently examined the effects of *R. schlippenbachii* extract on melanin synthesis and cellular tyrosinase activity.

3.2. Effect of *R. schlippenbachii* 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 the processes of growth and melanization are intimately related in melanoma cells (Huberman and Callaham, 1979; Siracký *et al*., 1984). Lan *et al*. (2005) reported that melanin content was significantly lower 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 *R. schlippenbachii* extract, the stimulatory effect of *R. schlippenbachii* extract on melanin was evaluated in B16 melanoma cells. The B16 melanoma cells were treated with the *R. schlippenbachii* extract at 5, 10, and 20 μ g/mL for 72 h. The melanin content was presented as a percentage of the control (vehicle). The following effects of *R. schlippenbachii* extract on melanogenesis of the B16 melanoma cells was found: *R. schlippenbachii* ethanol extract exerted a marked stimulatory effect on melanogenesis, without affecting cell proliferation, at concentrations of 5–20 μg/mL (Fig. 2). The *R. schlippenbachii* extract exhibited a significant

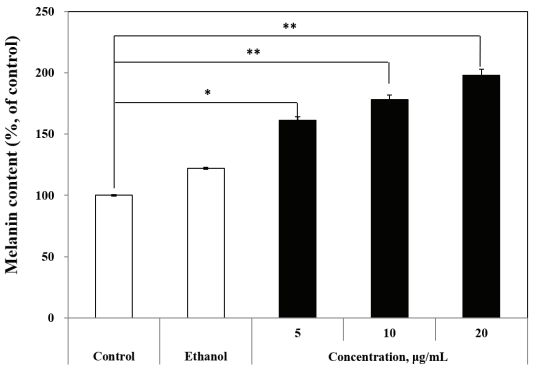


Fig. 2. Effect of *R. schlippenbachii* ethanol extract on melanin content in B16 melanoma cells. Control: untreated; Ethanol: 60 % ethanol; Concentration: *R. schlippenbachii* ethanol extract.

* $p<0.05$ compared to the untreated control.
** $p<0.01$ compared to the untreated control.

dose-dependent increase on melanin content. The melanin content was 142.11% ± 0.07%, 180.00% ± 0.10%, and 181.95% ± 0.38% after treatment with 5, 10, and 20 μg/mL *R. schlippenbachii* extract, respectively. Kang *et al*. (2018) reported that the melanin content was 146% and 110% after treatment with 8 μg/mL and 40 μg/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 C*. spinosa extract has the ability to stimulate 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 *R. schlippenbachii* extracts were confirmed to result in similar levels of melanin as previous studies, with a maximum melanin content of 181.95% after treatment with 20 μg/mL extract. Therefore, the *R. schlippenbachii* extract has the potential as a new natural resource to increase melanin content. 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. In previous paper, the colors of cell pellets were evaluated visually for melanin content assay (Usuki *et al*., 2003). Therefore, these observations suggest that the *R. schlippenbachii* extract increased the melanogenic activity of B16 melanoma cells.

3.3. Effect of *R. schlippenbachii* extract on mushroom tyrosinase activity

To determine whether *R. schlippenbachii* 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 *R. schlippenbachii* ethanol extract on mushroom tyrosinase activity are shown in Fig. 3. We observed that the effects of the extract on the oxidation of L-DOPA by mushroom tyrosinase occurred in a dose-dependent manner. At 10 μg/mL and 20 μg/mL, *R. schlippenbachii* ethanol extract exerted activity on the oxidation of L-DOPA by

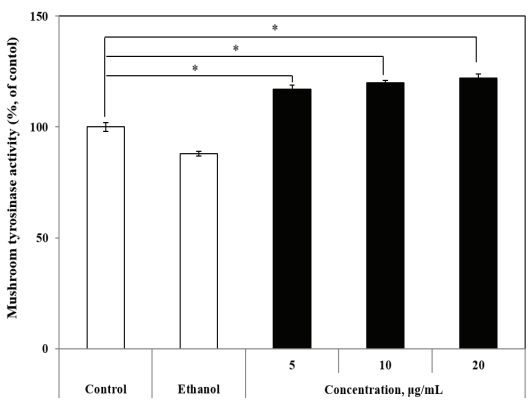


Fig. 3. Effects of *R. schlippenbachii* ethanol extract on mushroom tyrosinase activity. Purified tyrosinase was mixed with *R. schlippenbachii* ethanol extract and incubated with L-DOPA. Control: untreated; Ethanol: 60 % ethanol; Concentration: *R. schlippenbachii* ethanol extract.

mushroom tyrosinase; the 10 μg/mL *R. schlippenbachii* ethanol extract showed lower activity than the 20 μg/mL *R. schlippenbachii* 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- Cervantes *et al*., 1993). Therefore, melanin production is related to tyrosinase expression; our results also showed the similarity. More specifically, both the melanin content and tyrosinase activity were increased by 10 μg/mL and 20 μg/mL *R. schlippenbachii* extract (Fig. 2 and Fig. 3). Most studies have been performed on the effect of the mushroom tyrosinase from various plant extracts (Yoshimura *et al*., 2005; Kim *et al*., 2003). Thus, *R. schlippenbachii* extract may be noted as an effective material with mushroom tyrosinase activity.

3.4. Effect of *R. schlippenbachii* extract on tyrosinase activity in B16 melanoma cells

Tyrosinase catalyzes catalyze 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 *R. schlippenbachii* ethanol extract. Different concentrations of *R. schlippenbachii* 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 *R. schlippenbachii* 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 *R. schlippenbachii* extract (Fig. 4). Chen *et al*. (2012) showed that tyrosinase was regarded as the rate-limiting enzyme of melanogenesis,

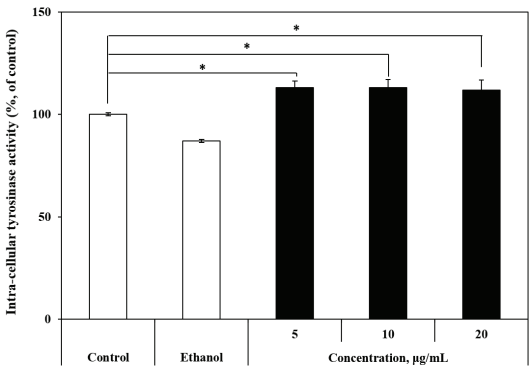


Fig. 4. Effect of *R. schlippenbachii* ethanol extract on intracellular tyrosinase activity in B16 melanoma cells (Control: untreated; Ethanol: 60% ethanol treated). * $p<0.05$ compared to the untreated control.

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) also 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, we found that *R. schlippenbachii* extract exerted a significant influence on tyrosinase activity. This result shows that the *R. schlippenbachii* 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 5–40 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 μg/mL extract clearly demonstrated a four-fold increase in intracellular tyrosinase activity (Hamid *et al.*, 2012). These results suggested that *R. schlippenbachii* extract can be used as a stimulant of tyrosinase, similar to other natural extracts, in previous studies; moreover, they found that melanin synthesis activated as tyrosinase activity increased.

3.5. Effect of *R. schlippenbachii* extract on melanin content in C57BL/6J Ler−*vit*/*vit* mice hair

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 *R. schlippenbachii* ethanol extract than in the control group ($p < 0.01$) (Fig. 5). Therefore, the *R. schlippenbachii* extract has the potential to assist repigmentation in vitiligo, because it increased melanin

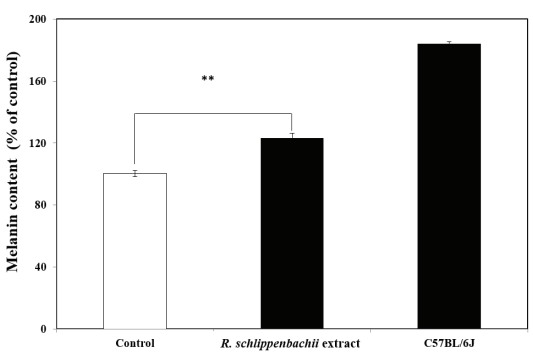


Fig. 5. Effect of *R. schlippenbachii* ethanol extract on melanin content of hair in C57BL/6J Ler-*vit*/*vit* mice. The mice hair was lysed with 1N NaOH and the absorbance of the solution at 500 nm was measured. Control: hair of C57BL/6J Ler-*vit*/*vit* mice treated 60% ethanol; *R. schlippenbachii* extract: hair of C57BL/6J Ler-*vit*/*vit* mice treated *R. schlippenbachii* ethanol extract; C57BL/6J: normal mice hair.
** $p<0.01$ compared to the control.

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 (Nogueira *et al*., 2007). However, the production of eumelanin is important for repigmentation in the treatment of vitiligo. The effect of *R. schlippenbachii* extract on the eumelanin content of mouse hair is shown in Fig. 6: treatment with *R. schlippenbachii* 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 *R. schlippenbachii* extract resulted in a lower eumelanin content than the hair of the normal C57BL/6J mice, we confirmed that the

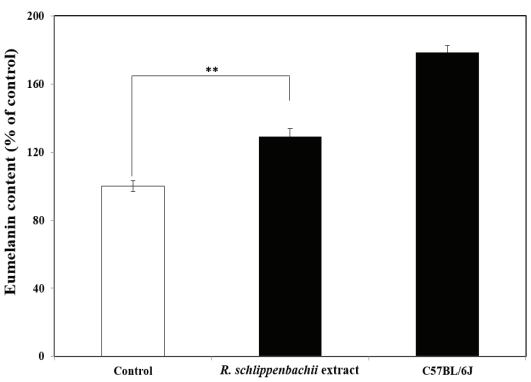
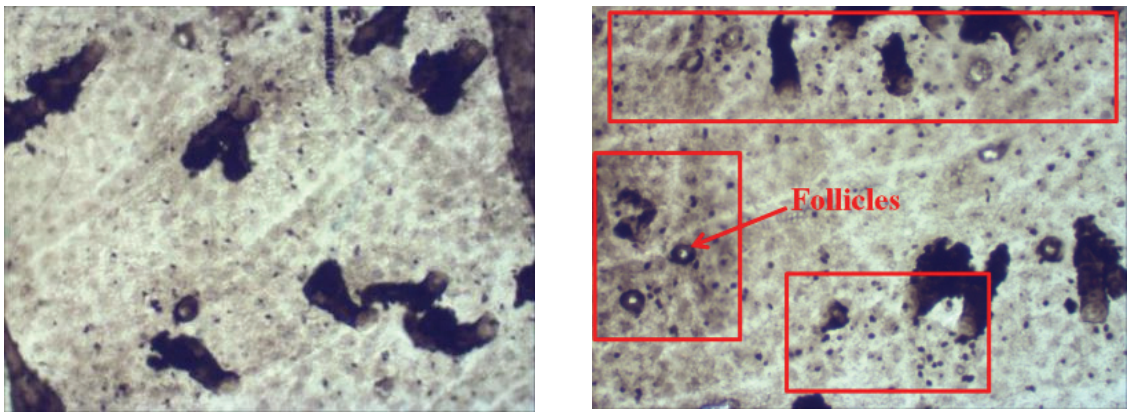


Fig. 6. Effect of *R. schlippenbachii* ethanol extract on eumelanin content of hair in C57BL/6J Ler-*vit*/*vit* mice. The mice hair was lysed with 1N NaOH and the absorbance of the solution at 650 nm was measured. Control: hair of C57BL/6J Ler-*vit*/*vit* mice treated 60% ethanol; *R. schlippenbachii* extract: hair of C57BL/6J Ler-*vit*/*vit* mice treated *R. schlippenbachii* ethanol extract; C57BL/6J: normal mice hair.
** $p<0.01$ compared to the control.

R. schlippenbachii 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 can affect not only the skin but also the head and other parts of the body (Mihăilă *et al*., 2019). Thus, we suggest that *R. schlippenbachii* extracts can be effectively used for repigmentation in the treatment of vitiligo.

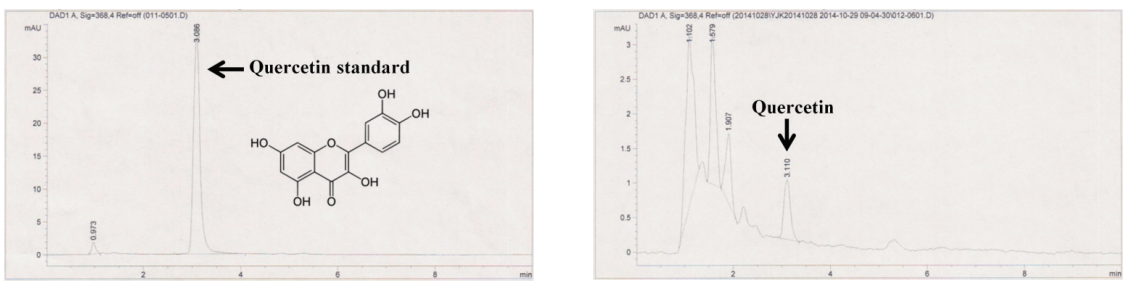
3.6. Effect of *R. schlippenbachii* extract on melanin cells in the histology of C57BL/6J Ler−*vit*/*vit* mice

Melanin cells around hair follicles observed under an optical microscope correspond, by their localization, to the DOPA-positive cells observed under the optical microscope. Their morphological features are characteristic of melanocytes. In Fig. 7, representative sections of C57BL/6J Ler-*vit*/*vit* mice skin stained for melanin are shown. Microscopic examination revealed pigmented areas, with more repigmentation after treatment with *R. schlippenbachii* ethanol extract than the 60% ethanol used as a control. As shown by the staining, *R. schlippenbachii* extract promoted a melanocytic response and the concentration required for the promotion of pigmentation was 0.2 mL/cm^2. These observations confirmed that *R. schlippenbachii* extract restored vitiligo pigmentation. Interestingly, *R. schlippenbachii* 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 tended to occur 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



Control *R. schlippenbachii*

Fig. 7. Histologic examination of *R. schlippenbachii* ethanol extract treated C57BL/6J Ler-*vit*/*vit* mice skin. (× 100). Control: C57BL/6J Ler-*vit*/*vit* mice treated 60% ethanol; *R. schlippenbachii*: C57BL/6J Ler-*vit*/*vit* mice treated *R. schlippenbachii* ethanol extract applied 0.2 mL/cm^2.



A B

Fig. 8. The content of quercetin of the standard and *R. schlippenbachii* extract by HPLC were shown. A: Quercetin standard (Retention time : 3.086 min), B: *R. schlippenbachii* extract.

repigmentation of the epidermis following dermabrasion originates from the melanotic portion of the hair follicle and it has been confirmed that *R. schlippenbachii* extract was an effective stimulator of the melanin stored around the hair follicles.

3.7. High-performance liquid chromatography (HPLC) detection of quercetin in *R. schlippenbachii* extract

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; Kwon *et al*., 2009; Lee *et al*., 2004). In addition, quercetin was previously reported to control the oxidation of l-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. We performed HPLC

analysis to determine if quercetin was contained in the *R. schlippenbachii* extracts that we produced. The application of HPLC to the study of quercetin in *R. schlippenbachii* extract is shown in Fig. 8: the *R. schlippenbachii* extract had the same retention time as the quercetin standard, which meant that quercetin was present in the *R. schlippenbachii* extract. This was similar to previous studies that reported quercetin was contained in *R. schlippenbachii* extracts (Glyzin *et al*., 1970). Therefore, it appears that quercetin plays a role in the melanogenic activity of *R. schlippenbachii* extracts. Further, we have identified the potential of *R. schlippenbachii* extract to be an effective source of quercetin for melanogenesis.

In this study, the effects of extract from *R. schlippenbachii* on melanogenesis and tyrosinase activity were evaluated in B16 melanoma cells and C57BL/6J Ler-*vit*/*vit* mice. The results clearly demonstrated the *R. schlippenbachii* extract enhanced melanogenesis and also increased tyrosinase activity in cultured melanoma cells and C57BL/6J Ler-*vit*/*vit* mice. It is anticipated that continued research will increase knowledge concerning the activity of tyrosinase in *R. schlippenbachii* ethanol extract, and continue to shed light on therapeutic strategies that can be used to reduce or eliminate vitiligo. Unfortunately, there is little research about the activity of the mushroom tyrosinase in plant extracts. In addition, treatment with *R. schlippenbachii* 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. Finally, we confirmed a notable increase in melanocytes in the skin of C57BL/6J Ler-*vit*/*vit* mice treated with *R. schlippenbachii* extract compared with the control. This study provides experimental evidence that *R. schlippenbachii* could be used as an effective treatment for the treatment of vitiligo and other skin diseases. However, further studies are necessary to establish specific details, such as how long the pigmentation lasts, what happens if the application is interrupted, how skin type and color influence the results, and effect for vitiligo treatment of other components excepted quercetin. In conclusion, *R. schlippenbachii* extract enhanced tyrosinase activities in mushroom tyrosinase and B16 melanoma cells. In addition, the extract was increased melanin content in melanocytes in both the B16 melanoma cells and C57BL/6J Ler-*vit*/*vit* mice hair. This study provides experimental evidence that *R. schlippenbachii* extract will be available as a therapeutic agent to relieve vitiligo.

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