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Inhibitory effect of *Rhinacanthus nasutus* (L.) Kurz leaf extract on melanogenesis in B16F10 melanoma cells

Benjamart Pratoomthai* Warachin Gangnonngiw**
Jarinyaporn Naowaboot*** Thanitsara Songtavisin****

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Background : *A previous study on the inhibitory effects of Rhinacanthus nasutus (L.) Kurz leaf ethanol extract on mushroom tyrosinase activity indicated that it contains anti-melanogenesis properties. However, other solvents especially water had not been investigated with regards to their use as a skin whitening agent.*

Objective : *To investigate the inhibitory effect R. nasutus leaf water extract on melanogenesis in B16F10 melanoma cells.*

Methods : *R. nasutus leaf was extracted with water and a determined amount of the phenolic and flavonoid contents by using Folin-Ciocalteu and aluminium chloride colorimetric assay, respectively. The toxicity of R. nasutus leaf extract in B16F10 melanoma cells was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Its potential on melanogenesis inhibition was determined by melanin content assay and tyrosinase activity assay. Data were analyzed by using SPSS.*

* Department of Basic Medical Science, Faculty of Medicine Vajira Hospital, Navamindradhiraj University
** Centex Shrimp, Faculty of Science, Mahidol University, and National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency
*** Division of Pharmacology, Faculty of Medicine, Thammasat University
**** Division of Anatomy, Faculty of Medicine, Thammasat University

Results : *R. nasutus leaf extract have a total phenolic content of 35.75 ± 0.30 mg gallic acid equivalents per gram of extract and flavonoid of 19.17 ± 0.00 mg rutin equivalent per of extract. We found that R. nasutus leaf extract decreased the melanin content and intracellular tyrosinase activity in B16F10 melanoma cells without any cytotoxicity.*

Conclusion : *Our results indicate that R. nasutus leaf water extract inhibited the melanin production by downregulating tyrosinase activity. Thus it might prove to be useful as a therapeutic treatment for skin hyperpigmentation disorders and an effective component of whitening cosmetics.*

Keywords : *Rhinacanthus nasutus, tyrosinase, melanin, melanogenesis.*

Correspondence to: Pratoomthai B. Department of Basic Medical Science, Faculty of Medicine Vajira Hospital, Navamindradhiraj University, Dusit, Bangkok 10300, Thailand.

E-mail: maew_199@hotmail.com

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เหตุผลของการทำวิจัย : *การศึกษาที่ผ่านมาได้รายงานว่าสารสกัดจากใบทองพันชั่งโดยใช้ตัวทำละลายเอทานอล มีความสามารถในการยับยั้ง mushroom tyrosinase activity ซึ่งบ่งชี้ถึงคุณสมบัติในการยับยั้งกระบวนการสร้างเมลานิน อย่างไรก็ตามยังไม่มีรายงานว่าสารสกัดโดยใช้ตัวทำละลายอื่น ๆ โดยเฉพาะอย่างยิ่ง น้ำ มีคุณสมบัติในการเป็นสารทำให้ผิวขาว*

วัตถุประสงค์ : *เพื่อศึกษาผลของสารสกัดจากใบทองพันชั่งโดยใช้น้ำเป็นตัวทำละลายต่อการยับยั้งการสร้างเมลานินในเซลล์ B16F10 melanoma*

วิธีการทำวิจัย : *สกัดใบทองพันชั่งด้วยน้ำ และศึกษาปริมาณสารประกอบ phenolic และ flavonoid โดยวิธี Folin-Ciocalteu และ aluminium chloride colorimetric assay ตามลำดับ ทดสอบความเป็นพิษต่อเซลล์โดยวิธี 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) ทดสอบความสามารถในการยับยั้งการกระบวนการสร้างเมลานิน โดยวิธี melanin content assay และ tyrosinase activity assay วิเคราะห์ข้อมูลโดยใช้โปรแกรม SPSS*

ผลการศึกษา : *พบว่าสารสกัดใบทองพันชั่งมีปริมาณ phenolic 35.75 ± 0.30 มิลลิกรัม/กรัม gallic acid equivalent และ flavonoid 19.17 ± 0.00 มิลลิกรัม/กรัม rutin equivalent ผลการทดลองพบว่า สารสกัดใบทองพันชั่งสามารถยับยั้งการสร้างเมลานิน และยับยั้งการทำงานของเอนไซม์ไทโรซิเนสโดยไม่มีฤทธิ์เป็นพิษต่อเซลล์*

สรุป : *ผลการทดลองแสดงให้เห็นว่าสารสกัดจากใบทองพันชั่ง สามารถยับยั้งการสร้างเมลานินโดยการลดการทำงานของเอนไซม์ไทโรซิเนส ดังนั้นอาจเป็นไปได้ที่จะมีการพัฒนาสารสกัดจากใบทองพันชั่งเพื่อนำมาใช้ในการรักษาความผิดปกติที่เกิดจากการสร้างเมลานินมากเกินไป และอาจจะนำมาใช้เป็นส่วนประกอบของเครื่องสำอางค์ประเภทผิวขาวใสได้*

คำสำคัญ : *ทองพันชั่ง ไทโรซิเนส เมลานิน กระบวนการสร้างเมลานิน.*

The appearance of mammal skin color is primary determined by a main pigment, melanin. This pigment is produced by melanocyte within the basal layer of the epidermis which responds to chemical, drug, or UV radiation stimuli. The biosynthesis process of melanin is called melanogenesis, which is regulated by many enzymes; however, only tyrosinase is considered as a key regulator in this process. Tyrosinase catalyses the hydroxylation of tyrosine to form 3,4-dihydroxy-phenylalaine (DOPA) and the oxidation of DOPA to produce DOPA-quinone before melanin is finally formed through a series of oxidation reactions.[1]

Although, melanin production has an important role in protecting the skin from UV radiation damage, excess biosynthesis is the cause of many hyperpigmentation disorders such as postinflammatory pigmentation, melasma, and skin-aging process.[2, 3]Therefore, many synthetic and natural compounds were used for treatment of these disorders. Synthetic compounds such as kojic acid, hydroquinone, and arbutin (hydroquinone-O-b-glucopyranoside) are available on the market as skin lighteners. However, kojic acid, is reported to cause skin irritation and dermatitis, and has carcinogenic potential. [4, 5] Hydroquinone and arbutin has a risk for causing exogenous ochronosis and perdurable depigmentation. [6, 7] Thus, the development of safe skin-whitening natural products has been sought after.

Rhinacanthus nasutus (L.) Kurz was distributed in many countries including Thailand. Previous studies reported that *R. nasutus* contains antioxidant, anti-fungal, anti-bacterial, anti-viral, anti-cancer, anti-inflammatory, anti-diabetic and anti-tumor activities. [8 - 13] A recent study found that *R. nasutus* leaf ethanol extract contains anti-melanogenesis properties by inhibiting mushroom tyrosinase activity. [14] Hence in the present study, we demonstrated the skin-whitening potential of *R. nasutus* leaf water extract in particular on inhibition of melanogenesis in B16F10 melanoma cells.

Materials and Methods

Chemicals and Reagents

α-melanocyte-stimulating hormone (α-MSH), kojic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-3,4-dihydroxyphenylalanine (L-DOPA), dimethyl sulphoxide (DMSO) and synthetic melanin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), trypsin, penicillin, and streptomycin were purchased from Gibco/BRL (Scotland, UK).

Rhinacanthus nasutus (L.) Kurz leaf extracted

R. nasutus leaves (100 g) were collected from northeast of Thailand in September 2016. The samples were washed and air-dried. The dried leaves were extracted with water at 100 ^{o}C for 30 min and filtered. The extract was then concentrated and freeze-dried. After this procedure, the yield was 25.11% of the initial dry weight of the leaves. The obtained *R. nasutus* leaf extract was kept at -20 ^{o}C until it was ready for further use. The amount of the total phenolic and flavonoid contents were determined using the Folin-Ciocalteu method and the aluminium chloride colorimetric assay [15, 16] respectively.

Mushroom tyrosinase activity assay

Mushroom tyrosinase activity was determined

using the method described previously [17] with some modifications. Briefly, 20 μl of the test sample and 160 μl of 5 nM L-DOPA in 0.1 M sodium phosphate buffer (PBS) pH 6.8 were placed into the wells of a 96-well plate after which was added 20 μl of mushroom tyrosinase (200 units/ml) and then incubated at 37 ^{o}C for 30 min. The absorbance was measured at 475 nm using a microplate reader (TECAN, Mannedorf, Switzerland).

Cell culture

B16F10 mouse melanoma cell line obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dubecco's Modified Eagle Medium (DMEM) (ATCC, Manassas, VA, USA) with 10% FBS, and 1% penicillin/streptomycin (10,000U/100 mg/ml) at 37 ^{o}C in 5% CO_2 humidified air. The cells (passage 5-7) were trypsinized, sub-cultured and treated with various concentrations of tested samples for 72 h.

Cell viability assay

Cell viability was determined using MTT-based assay. [18] B16F10 melanoma cells (2.5 x 10^3 cells/well) were cultured in 96-well plates with various concentrations of *R. nasutus* leaf extract (5, 10, 20, 50, 100 μg/ml) or kojic acid (100 μg/ml), used as a positive control, for 72 h. After treatment, a culture medium was removed and 0.5 mg/ml of MTT (dissolved in PBS) solution was added for 3 h at 37 ^{o}C. The supernatant was discarded and then DMSO was added. After incubation at 37 ^{o}C for 15 min, the optical density was measured at 490 nm using a microplate reader (TECAN, Mannedorf, Switzerland).

Melanin content and microscopy

Intracellular melanin content was determined using the method described previously [19] with some modifications. Briefly, B16F10 melanoma cells (2.5 x 10^4 cells/well) were seeded into a 6-well plate and incubated for 24 h before being treated with various concentrations of *R. nasutus* leaf extract (5, 10, 20, and 50 μg/ml) or kojic acid (100 μg/ml) in the presence of 100 nM of α-MSH for 72 h. After treatment, cell pellets were dissolved in 1 ml of 1 N NaOH at 100 ^{o}C for 30 min. The melanin content was measured at 405 nm using a microplate reader (TECAN, Mannedorf, Switzerland). Cells were also observed under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan).

Intracellular tyrosinase activity

Intracellular tyrosinase activity was determined using the method described previously [20] with some modifications. Briefly, B16F10 melanoma cells (2.5x 10^4 cells/well) were seeded into a 6-well plate and incubated for 24 h before being treated with various concentrations of *R. nasutus* leaf extract (5, 10, 20, and 50 μg/ml) or kojic acid (100 μg/ml) in the presence of 100 nM of α-MSH for 72 h. After treatment, cells were washed with cold PBS and lysed with PBS containing 1% Triton-X and then frozen at -80 ^{o}C for 30 min. After thawing, the lysates were centrifuged and the protein concentration was determined by using the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA). Then 100 μg protein lysates (adjusted to 100 μl withPBS) and 100 μl of 5 mM L-DOPA were placed into a 96-well plate. After incubation at 37 ^{o}C for 1 h, the absorbance was measured at 475 nm using a microplate reader (TECAN, Mannedorf, Switzerland).

Statistical analysis

All data were carried out in triplicate and results were presented as mean ± standard deviation (SD). Statistical analyses were performed by one way analysis of variance (ANOVA) complemented by Dunnett's post hoc test. Differences were considered significant when the *P* value was less than 0.05 by utilizing the SPSS statistical analysis software (SPSS Inc., Chicago, IL, USA).

Results

Total phenolic and flavonoid contents

R. nasutus leaf extract has a total phenolic content of 35.75 ± 0.30 mg gallic acid equivalents per gram of extract and flavonoid of 19.17 ± 0.00 mg rutin equivalent per of extract.

Cytotoxicity of R. nasutus leaf extract in B16F10 melanoma cells

To investigate whether tested samples have cytotoxic effects, B16F10 melanoma cells were treated with various concentrations of *R. nasutus* leaf extract or kojic acid. As shown in Figure 1, treatment with *R. nasutus* leaf extracts did not affect cell viability up to 50 μg/ml; however, at concentrations of 100 μg/ml, cytotoxicity was observed. There was an approximately 20% decrease in cell viability at 100 μg/ml. Thus, *R. nasutus* leaf extracts at a concentration ≤ 50 μg/ml were used to determine their effect on melanogenesis in B16F10 melanoma cells.

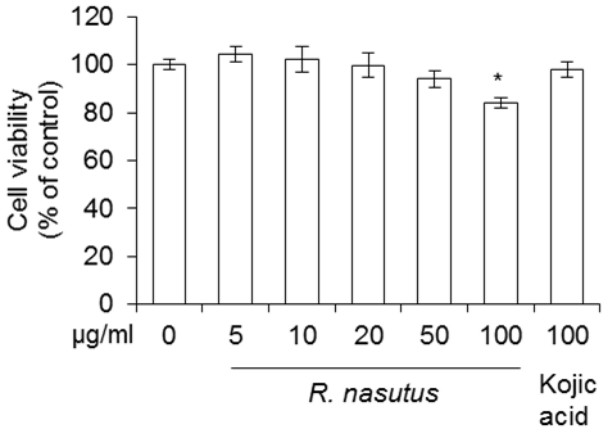

Figure 1. Effect of *R. nasutus* leaf extract and kojic acid on cell viability in B16F10 melanoma cells. Baseline cell viability in control wells not exposed to *R. nasutus* leaf extract or kojic acid was set at 100%. Data were expressed as percentage of control. Each column represents the mean ± SD of three independent experiments.

Effect of R. nasutus leaf extract on melanin production

The inhibitory effect of *R. nasutus* leaf extract on melanin production was investigated by intracellular melanin content assay. The melanin contents of B16F10 melanoma cells treated with α-MSH increased 68% higher than the negative control group (without α-MSH). Cells cultured with increasing concentrations of *R. nasutus* leaf extract (5, 10, 20, and 50 μg/ml) showed levels of melanin content decreasing by 21, 44, 57 and 77%, respectively (Figure 2). Kojic acid (100 μg/ml) reduced the melanin level in cells by 75%. According to the phase contrast results, melanin content in *R. nasutus* leaf extract- and kojic acid treated cells was less than that of the control group (Figure 3).

Effect of R. nasutus leaf extract on tyrosinase activity

To determine whether *R. nasutus* leaf extract has an effect on tyrosinase activity directly or not, mushroom tyrosinase activity assay was performed. The results showed that *R. nasutus* leaf extract did not inhibit mushroom tyrosinase activity (Figure 4A), whereas kojic acid, inhibited mushroom tyrosinase activity in a dose-dependent manner (Figure 4B). These results confirm the inhibitory effects of kojic acid on tyrosinase acitivity. However, when tested on cellular tyrosinase activity, results showed a significant decrease of tyrosinase activity after treatment with *R. nasutus* leaf extract with presence of α-MSH (Figure 4C).

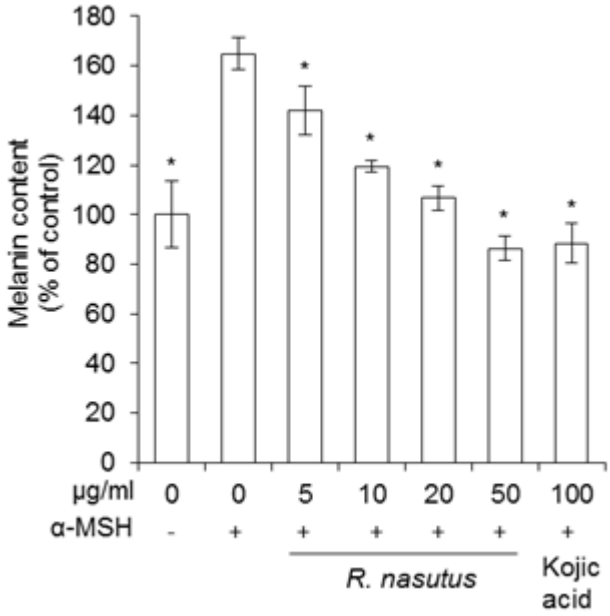

Figure 2. Effect of *R. nasutus* leaf extract on melanin content in B16F10 melanoma cells. Baseline melanin content in control wells not exposed to α-MSH or *R. nasutus* leaf extract or kojic acid was set at 100%. Data were expressed as percentage of control. Each column represents the mean ± SD of three independent experiments. * indicates value significantly different from the α-MSH treatment group ($P < 0.05$).

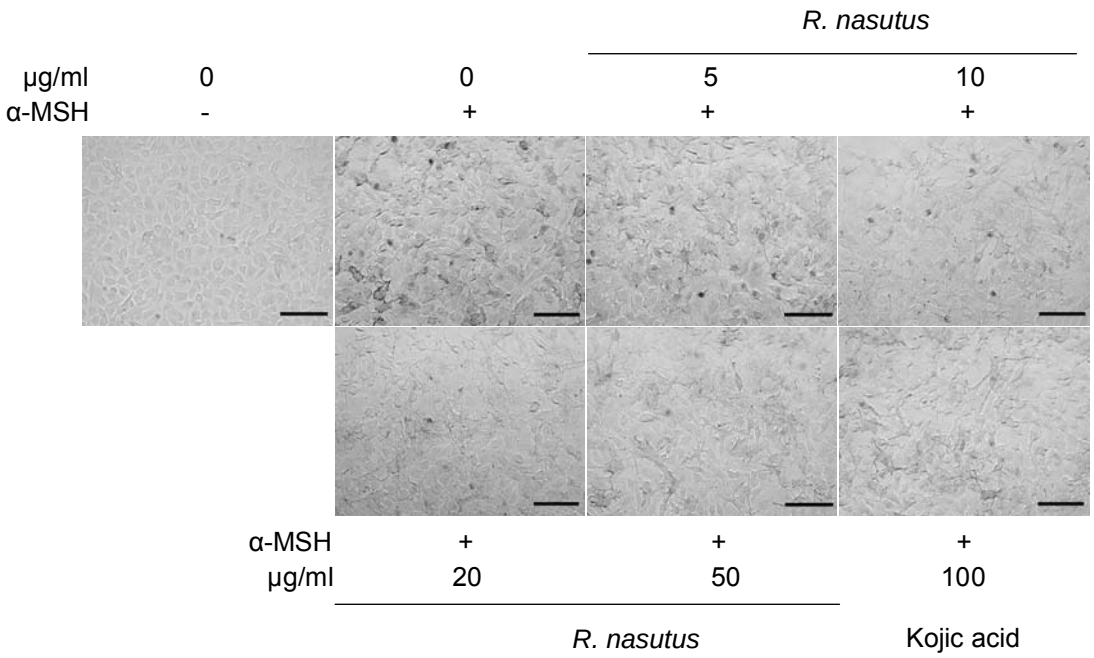

Figure 3. Effect of *R. nasutus* leaf extract and kojic acid on melanin content in B16F10 melanoma cells. Melanin content (dark pigment) was observed under a phase contrast microscopy. Scale bar = 100 μm

Discussion

The purpose of this study was to investigate the possible skin-whitening property of *R. nasutus* leaf extract by focusing on its inhibition of melanogenesis in B16F10 melanoma cells. B16F10 melanoma cells are widely used as a model for screening the inhibition of compounds on melanogenesis because they are easy to culture and share most of the melanogenic mechanisms of normal human melanocytes. [5] In addition, kojic acid was used as a positive control because it is known as skin whitening agent in cosmetics. [21] In this study, we also used α-MSH as a melanogenesis stimulator. In general, α-MSH is released from keratinocyte after the stimulation of UV radiation. This hormone binds to its receptor on melanocyte that leads to the stimulation of tyrosinase before the melanin is produced. [22]

We found that *R. nasutus* leaf extract inhibits melanin production in cells without any cytotoxicity at dose for testing, proving the potential properties of this compound on melanogenesis inhibition. In order to evaluate the possible mechanisms by which *R. nasutus* leaf acts, its effect on tyrosinase activity was investigated. Inhibition of tyrosinase activity is the most common target for melanogenesis inhibitors. In the view of clinical applications in cosmetics and pharmaceuticals, tyrosinase inhibitors are most popular and wildly used as hypopigmentation agents. Because tyrosinase is produced only by melanocytic cells, tyrosinase inhibitors specifically target melanogenesis in the cells without other side effects. In the present study, direct inhibitory effect of *R. nasutus* leaf extract on tyrosinase activity was observed in mushroom tyrosinase activity assay.

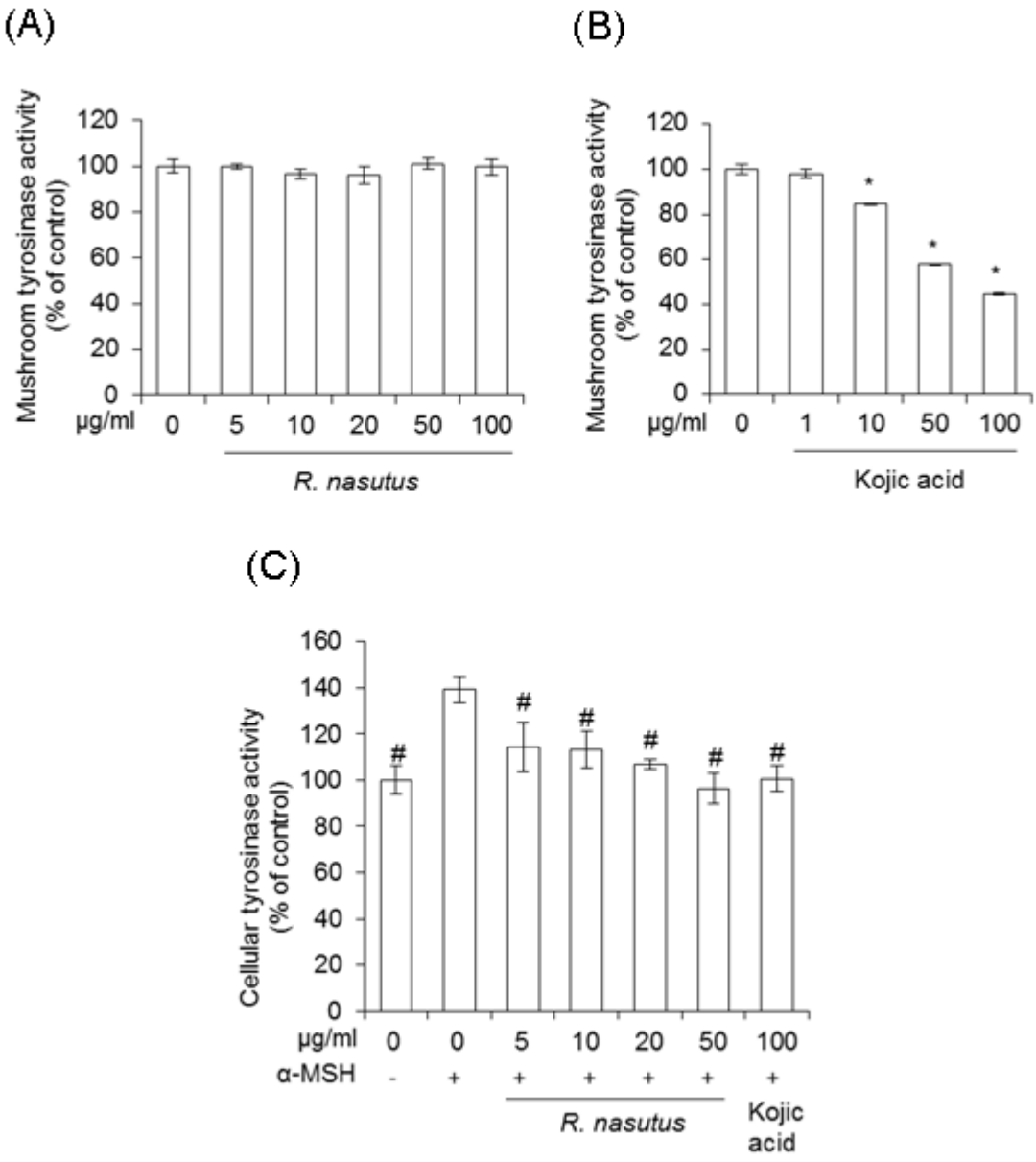

Figure 4. Effect of *R. nasutus* leaf extract on mushroom tyrosinase activity (A) compared with that of kojic acid, a positive control (B), and tyrosinase activity in B16F10 melanoma cells (C). Baseline tyrosinase activity in control wells not exposed to α-MSH or *R. nasutus* leaf extract or kojic acid was set at 100%. Data were expressed as percentage of control. Each column represents the mean ± SD of three independent experiments. * indicates value significantly different from the control ($P < 0.05$). # indicates value significantly different from the α-MSH treatment group ($P < 0.05$).

Mushroom tyrosinase is a commercially available enzyme that is often used as a substitute for human tyrosinase in order to perform screenings for tyrosinase inhibitors. [23] We found that *R. nasutus* water extract did not exhibit any inhibitory effects on mushroom tyrosinase activity. Our results contrast with a previous study that reported *R. nasutus* ethanol extract inhibited the mushroom tyrosinase activity. [14] The reason for the difference in results is still unknown; however, it is possible that there are other different substances

could be extracted by ethanol that can inhibit mushroom tyrosinase activity and those substances could be more effective than phenolics and flavonoids. However, we found that *R. nasutus* leaf extract inhibited cellular tyrosinase activity. From these results it could be possible that the inhibition of *R. nasutus* leaf extract on melanogenesis might not be due to direct inhibition of tyrosinase, but rather inhibition via intracellular signaling pathways. In the present study the mechanisms by which *R. nasutus* leaf extract inhibit the melanogenesis remains unknown and should be further investigated.

Several studies have reported the inhibitory effects of phenolics and flavonoid on melanogenesis from various sources including soybeans,*Ornithogalum narbonense*, *Mentha*, and mushrooms. [24-27] In this study we found a high level of the phenolic and flavonoid contents in *R. nasutus* leaf extract, therefore, it is possible that the inhibitory effects on melanin production and tyrosinase activity may result from the contained-chemical compounds including flavonoids and phenolics. However, the purified compounds are other subjects for further study.

Conclusion

The present study found that *R. nasutus* leaf extract inhibited melanin production and tyrosinase activity in B16F10 mouse melanoma cells. The results suggest that *R. nasutus* leaf extract is a potential anti-melanogenic agent that could be applied as an active skin-whitening ingredient in cosmetics or as a topical agent for the treatment of hyperpigmentation disorders.

Conflict of interest

The authors, hereby, declare no conflict of interest.

Acknowledgment

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