# Assessment of the Effect of Arbutin Isomers and Kojic Acid on Melanin Production, Tyrosinase Activity, and Tyrosinase Expression in B16-4A5 and HMV-II Melanoma Cells

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# Abstract

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The inhibitory effects of  $\alpha$ -arbutin,  $\beta$ -arbutin, and kojic acid on melanogenesis, tyrosinase activity, and tyrosinase protein expression in mouse melanoma cells (B16-4A5) and human melanoma cells (HMV-II) were directly compared.  $\beta$ -Arbutin showed a stronger inhibitory effect on melanogenesis and tyrosinase expression in B16-4A5 cells than  $\alpha$ -arbutin and kojic acid. Kojic acid showed a stronger inhibitory effect on mushroom and B16-4A5 tyrosinase activity than  $\alpha$ -arbutin and  $\beta$ -arbutin. In contrast, kojic acid inhibited all of these effects more strongly than  $\alpha$ -arbutin or  $\beta$ -arbutin in HMV-II cells. These results suggest that kojic acid may be used as a positive control for the inhibitory melanogenesis assay, and for tyrosinase activity and tyrosinase expression assays that use HMV-II cells. Moreover, using HMV-II cells with kojic acid as the positive control may facilitate the search for new skin-whitening agents using natural products and provide an alternative to the B16-4A5 assay.

**Key words** 

 $\alpha$ -arbutin ·  $\beta$ -arbutin · kojic acid · melanin · tyrosinase

**Supporting information** available online at http://www.thieme-connect.de/products

The production of excessive pigmentation in melasma, spots, freckles, and other forms of hyperpigmentation can pose a significant aesthetic problem [1,2]. The causative pigment, melanin, is formed by a combination of enzymatically catalyzed chemical reactions. Melanogenesis is initiated by the catalytic oxidation of tyrosine to dopaquinone by tyrosinase; this is the rate-limiting step in melanin synthesis [3]. Tyrosinase inhibitors reduce or block melanin synthesis, leading to skin whitening. Several common skin-lightening and depigmentation agents available commercially include arbutin (hydroquinone  $\beta$ -D-glucopyranoside,  $\beta$ -ARB), kojic acid (KA), and hydroquinone [4,5]. These compounds are isolated from natural resources and are bioassayed using mushroom tyrosinase and B16 mouse melanoma cells. In a recent study, human melanoma HMV-II cells were used as an alternative to B16 [6,7].  $\alpha$ -Arbutin ( $\alpha$ -ARB), the epimer of  $\beta$ -ARB (**•** Fig. 1), has inhibitory effects on melanogenesis and tyrosinase [8,9]. However, it remains unclear whether  $\beta$ -ARB or  $\alpha$ -ARB has strong activity against HMV-II, and the inhibitory effects of KA on melanogenesis and tyrosinase using HMV-II are also unclear.



In this study, we directly compared the inhibitory effects of these compounds on melanogenesis and tyrosinase activity using B16 and HMV-II.

The inhibitory effect of  $\alpha$ -ARB,  $\beta$ -ARB, and KA on the proliferation and pigmentation of B16-4A5 and HMV-II were compared. • Fig. 2A shows that  $\alpha$ -ARB,  $\beta$ -ARB, and KA at 125 to 1000  $\mu$ M suppressed melanogenesis in B16-4A5. β-ARB significantly suppressed melanogenesis in a concentration-dependent manner between 125 and 1000 μM, α-ARB significantly suppressed melanogenesis in a concentration-dependent manner between 250 and 1000 µM, whereas KA only significantly suppressed melanogenesis at 1000  $\mu$ M. These results showed that  $\beta$ -ARB suppressed melanogenesis more effectively than  $\alpha$ -ARB and KA in B16-4A5. • Fig. 2B shows that  $\alpha$ -ARB,  $\beta$ -ARB, and KA at 125 to 1000  $\mu$ M suppressed melanogenesis in HMV-II, KA significantly suppressed melanogenesis in a concentration-dependent manner between 125 and 1000  $\mu$ M, whereas  $\alpha$ -ARB and  $\beta$ -ARB significantly suppressed melanogenesis in a concentration-dependent manner between 500 and 1000 µM. These results demonstrated that KA was superior compared to  $\alpha$ -ARB and  $\beta$ -ARB at suppressing melanogenesis in HMV-II. The inhibitory effects of these compounds on melanogenesis in HMV-II were, thus, in contrast with their inhibitory effects on melanogenesis in B16-4A5. Significant cytotoxic effects were only observed for  $\alpha$ -ARB at 1000  $\mu$ M on B16-4A5,  $\beta$ -ARB at 500 and 1000  $\mu$ M on B16-4A5, and  $\beta$ -ARB at 1000 µM on HMV-II (Fig. 1 S, Supporting Information).

The inhibition of tyrosinase or/and downregulation of tyrosinase expression are mechanisms by which depigmentation occurs [3, 10]. Mushroom tyrosinase, B16-4A5, and HMV-II are used to screen for inhibitors of tyrosinase and suppressors of melanin biosynthesis [3, 4, 11]. • **Table 1** shows the inhibitory effect of  $\alpha$ -ARB,  $\beta$ -ARB, and KA on mushroom, B16-4A5, and HMV-II tyrosinase.  $\alpha$ -ARB interestingly showed an inhibitory effect on B16-4A5 tyrosinase (297.4 ± 9.7 µM) but no inhibitory effect on mushroom and HMV-II tyrosinases (> 500 µM). In contrast,  $\beta$ -ARB showed weak inhibition of all three tyrosinases (> 500 µM). The KA IC<sub>50</sub> inhibition values of mushroom, B16-4A5, and HMV-II tyrosinases were 297.4 ± 9.7 µM, 57.8 ± 1.8 µM, and 223.8 ± 4.9 µM, respectively.

We postulated that the melanogenesis inhibitory effect of KA might have contributed to the inhibition of tyrosinase. To investigate the mechanism by which  $\alpha$ -ARB,  $\beta$ -ARB, and KA inhibit melanogenesis, we used Western blotting to examine the effect of these compounds on the expression of B16-4A5 and HMV-II tyrosinase proteins (**© Fig. 3**). In B16-4A5, tyrosinase expression was decreased by  $\beta$ -ARB,  $\alpha$ -ARB, and KA (all 500  $\mu$ M, listed in order of

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decreasing effect) and this ordering of the compounds is the same as the order of their effect on reducing melanin in B16-4A5. In HMV-II, tyrosinase expression was decreased by KA,  $\beta$ -ARB, and  $\alpha$ -ARB (all 1000  $\mu$ M, listed in order of decreasing effect),



**Fig. 2** Inhibitory effect of  $\alpha$ -ARB,  $\beta$ -ARB, and KA on melanogenesis in B16-4A5 (**A**) and HMV-II (**B**) cells. Each compound at 125, 250, 500, or 1000  $\mu$ M was individually added to the cells on the first and fourth day of cell culture. On day 7, the number of viable cells in each culture was determined using 4% Alamar Blue reagent. Each column represents the mean ± SD of four independent tests (Student's t-test). \*P < 0.01, significantly different from the control value.

which is the same order as their effect on reducing melanin content. KA is a known tyrosinase inhibitor, but it does not decrease tyrosinase expression in several B16 cell lines [12]. However, there are no previous reports directly comparing the effect of KA with other agents in decreasing tyrosine levels in HMV-II, as apposed to B16 tyrosinase levels. The present data confirmed that the inhibition of melanogenesis by KA is due to a specific inhibition and downregulation of HMV-II tyrosinase.

In conclusion, KA directly inhibited tyrosinase activity and suppressed the expression levels of tyrosinase in HMV-II. Furthermore, this is the first report to directly compare the effects of  $\alpha$ -ARB,  $\beta$ -ARB, and KA on melanogenesis and to evaluate their inhibitory effect on tyrosinase in B16-4A5 and HMV-II. These results suggest that KA can be used as a positive control in the assessment of melanogenesis, tyrosinase activity, and tyrosinase expression in HMV-II cells, thereby providing an alternative to the mushroom tyrosinase assay and B16-4A5 assay in the search for new skin-whitening agents derived from natural products (**© Table 2**).

# **Materials and Methods**

*Cell cultures:* B16-4A5 (RCB0557) and HMV-II (RCB0777) cells were obtained from the RIKEN BRC through the National Bio-Resource Project of the MEXT, Tsukuba, Japan. B16-4A5 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences), penicillin, and streptomycin at 37°C in a humidified atmosphere consisting of 5% CO<sub>2</sub> and 95% air.

**Table 1** IC<sub>50</sub> values ( $\mu$ M) of the tyrosinase activity inhibitory effect of  $\alpha$ -ARB,  $\beta$ -ARB, and KA.

	Mushroom tyrosinase	B16-4A5 tyrosinase	HMV-II tyrosinase
α-ARB	> 500	$297.4 \pm 9.7$	> 500
$\beta$ -ARB	> 500	> 500	> 500
KA	182.7 ± 9.8	57.8 ± 1.8	223.8 ± 4.9

Values for each compound represent the mean ± SD of four independent tests

 Table 2
 Potential candidates for bioassays for new skin-whitening agents extracted from natural products.

	Mushroom	B16-4A5	HMV-II
Melanin production	-	$\beta$ -ARB	KA
Tyrosinase activity	KA	KA	KA
Tyrosinase expression	-	$\beta$ -ARB	KA



**Fig. 3** Inhibitory effect of  $\alpha$ -ARB,  $\beta$ -ARB, and KA on melanin production in B16-4A5 and HMV-II cells by downregulation of tyrosinase expression. The cells were treated with the compounds for 72 h. The expression levels of tyrosinase were examined by Western blot using antibodies.

HMV-II cells were cultured in Ham's F12 supplemented with 10% FBS (Vitromex), penicillin, and streptomycin at 37 °C in a humidified atmosphere consisting of 5%  $CO_2$  and 95% air.

*Measurement of melanin content:* The amount of melanin in cultured melanoma cells was measured as described previously [13, 14]. See Supporting Information for detailed protocols.

*Tyrosinase inhibition assay:* Mushroom tyrosinase activity was measured by determining its DOPA-oxidase activity using a modification of the method of Ha et al. [15]. First, 120  $\mu$ L of L-DOPA (8 mM, dissolved in 50 mM phosphate buffer, pH 6.8) and 40  $\mu$ L of the sample were mixed. Then, 40  $\mu$ L of mushroom tyrosinase (80 units/mL) was added, and the amount of dopachrome in the reaction mixture was determined by measuring the optical density at 492 nm after 20 min at 37 °C.

*Expression of tyrosinase in B16-4A5 and HMV-II cells:* Crude tyrosinase was prepared based on the method of Ohguchi et al. [16]. Tyrosinase expression was confirmed by Western blotting [17]. See Supporting Information for detailed protocols.

# Supporting information

Detailed information on materials and methods are available as Supporting Information.

Acknowledgements

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# **Conflict of Interest**

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The authors declare no conflict of interest.

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