

Anti-Tyrosinase Properties Of Different Species of Turmeric And Isolation of Active Compounds From Curcuma Amada

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Abstract

Turmeric is traditionally used as a skin cosmetic in some religious and cultural occasions on the Indian subcontinent. In this study, we compared the tyrosinase inhibitory properties of four *Curcuma* spp., namely, *C. xanthorrhiza*, *C. aromatica*, *C. amada*, and *C. zedoaria*. Bioassay-guided isolation and purification of tyrosinase inhibitors using silica gel column and high-performance liquid chromatography. Structural identification of the compounds was conducted using ^1H NMR, ^{13}C NMR, and liquid chromatography-tandem mass spectrometry. *C. amada* showed the highest tyrosinase inhibitory activity, with an IC_{50} of 53.4 $\mu\text{g}/\text{mL}$. Therefore, it was chosen for the isolation and purification of tyrosinase inhibitors. The purified compounds were zederone (**1**), furanodienone (**2**), 1,5-epoxy-3-hydroxy-1-(3,4-dihydroxy-5-methoxyphenyl)-7-(4-hydroxyphenyl) heptanes (**3**), 3,5-dihydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl) heptanes (**4**) and 1,5-epoxy-3-hydroxy-1-(3,4-dihydroxy-5-methoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl) heptanes (**5**). The IC_{50} values for the mushroom anti-tyrosinase activity of compounds **1**, **2**, **3**, **4**, and **5** were 108.2, 89.2, 92.3, 21.7 and 41.3 μM , respectively. These compounds also inhibited intracellular tyrosinase activity, thus reducing melanin synthesis in B16F10 melanoma cells. Compound **4** showed significantly stronger anti-tyrosinase activity than that of arbutin (a positive control drug). No significant difference was observed in the tyrosinase inhibitory effect between compound **5** and arbutin. Our findings strongly suggest that *C. amada* is a promising source of natural tyrosinase inhibitors to prevent melanogenesis and could be used as a whitening cosmetic.

Introduction

Melanin is the black pigment in hair and skin and is essential for protecting skin against UV radiation. The pigment is produced by melanocyte cells, present in the basal layer of the dermis through a physiological process called melanogenesis. However, abnormal production of melanin causes dermatological disorders such as freckles, melasma, lentigines, age spot, ephelide and post-inflammatory hyperpigmentation [1]. In the food industry, hyperpigmentation of fruits and vegetables leads to significant losses of nutritional quality and market value [2]. Melanogenesis can be controlled by inhibiting the activity of tyrosinase, a rate-limiting enzyme for melanin synthesis in mammals, plants, microorganisms and fungi [3]. Therefore, inhibition of tyrosinase activity prevents hyperpigmentation and leads to skin whitening. It also controls the quality of vegetables and fruits by regulating the undesired browning of vegetables and food.

Most skin-lightening agents, such as hydroquinone, azelaic acid, kojic acid and arbutin are potent tyrosinase inhibitors. However, they have various undesirable effects such as cytotoxicity, ochronosis, vitiligo, irritation, skin peeling, and redness [4]. Moreover, kojic acid and α -arbutin demonstrate poor formulation stability and skin penetration ability and low efficacy *in vivo* [5]. Some organic and inorganic mercury salts have anti-melanogenic effect and are used in skin-whitening agents. However, through cutaneous absorption, mercurial compounds can cause toxic effects such as skin discoloration, kidney damage, allergic reaction and scarring [6]. Thus, the investigation of less toxic and more effective tyrosinase inhibitors is needed.

Turmeric (family: Zingiberaceae; genus: *Curcuma*), a traditional medicinal plant that grows predominantly in the tropical and subtropical regions of Asia and Africa, has a broad spectrum of pharmacological functions. It has been traditionally used in premarital rituals for thousands of years in the Indian subcontinent as a skin lightening agent. Turmeric is believed to improve skin complexion by reducing facial hair growth, acne, and skin aging [7–8]. Therefore, skin care products supplemented with turmeric are commercially available in the market [9]. More than 70 species/varieties of turmeric with different chemical and pharmacological properties have been identified. However, there is a lack of scientific information on the anti-melanogenic properties of different species of turmeric and the potential active components present in turmeric. Curcuminoids are the main active compounds responsible for the majority of the biological activities of turmeric. Curcuminoids have potential in cosmeceuticals as antioxidant, anti-inflammatory and skin-lightening agents [7–8]. However, in a previous study, we found significant variations in curcuminoid content in turmeric, and some species (*C. amada*, *C. zedoaria*) did not even contain curcumin [10]. We also reported the antifungal, antioxidant and vasodilatory activities of different species and varieties of turmeric [10–13]. Therefore, the aims of this study were to evaluate the effects of different species of turmeric, namely, *C. xanthorrhiza*, *C. aromatica*, *C. amada* and *C. zedoaria*, on the tyrosinase enzyme and to identify the specific chemical compounds responsible for anti-tyrosinase activity. We also evaluated the tyrosinase inhibitory activity and anti-melanogenic effects of purified active compounds on melanin synthesis in B16F10 melanoma cells.

Results And Discussion

Among the four different turmeric species, the MeOH extract of *C. amada* showed the maximum mushroom tyrosinase inhibitory effect with an IC_{50} value of 53.4 ± 2.7 , followed by *C. xanthorrhiza*, *C. aromatica* and *C. zedoaria* (Fig. 2a). Curcumin is the major active component of turmeric (*Curcuma longa*) and possesses a wide range of biological activities, including anti-cancer, anti-inflammatory, anti-bacterial, anti-fungal, and anti-oxidant activities. It showed 75-fold more potent anti-tyrosinase activity than arbutin and kojic acid [14]. However, in a previous study, we reported that there were significant variations in curcumin contents in different species of turmeric [10]. Curcumin was present in *C. xanthorrhiza* and *C. aromatica* but absent in *C. zedoaria* and *C. amada* [10]. The interesting findings of this study are that, without curcumin, *C. amada* showed strong tyrosinase inhibitory activity. This result indicates that there must be some active compounds in *C. amada* attributed to its strong anti-tyrosinase effect. The anti-tyrosinase activity of *C. xanthorrhiza* and *C. aromatica* might be due to their curcumin contents. However, we could not rule out the possibility of the presence of other compounds.

The MeOH extract of *C. amada* were fractioned with water, *n*-hexane and EtOAc. Among these three fractions, EtOAc showed a significantly stronger inhibitory effect than water and *n*-hexane (Fig. 2b). Therefore, the EtOAc part was taken for further fractionation. The anti-tyrosinase activities of six fractions [*n*-hexane:EtOAc; 100:0 (F1), 80:20 (F2), 60:40 (F3), 40:60 (F4), 20:80 (F5) and 0:100 (F6)] obtained from the EtOAc part of *C. amada* were compared. Among these six fractions, F6 and F3 showed significantly higher anti-tyrosinase activity than the others (Fig. 2c). Then the chemical structures of the five

compounds from fractions F3 and F6 were identified according to their ^1H NMR and ^{13}C NMR spectra. Peak data were as follows:

Compound 1: Colorless needle shape crystal; UV λ_{max} : nm 234, 285. ESI-MS (+) m/z : 247.3 $[\text{M} + \text{H}]^+$, 229.4 $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$. ^1H -NMR (CD_3OD): δ 7.22 (1H, s, H-12), 5.59 (1H, br d, $J = 12$ Hz, H-1), 3.99 (1H, s, H-5), 3.85 (1H, d, $J = 16$ Hz, H-9a), 3.69 (1H, d, $J = 16$ Hz, H-9b), 2.57 (1H, dddd, $J = 13, 13, 12, 4$ Hz, H-2a), 2.26 (1H, m, H-3a), 2.20 (1H, m, H-2b), 2.09 (3H, s, H-13), 1.57 (3H, s, H-15), 1.32 (1H, ddd, $J = 13, 13, 4$ Hz, H-3b), 1.28 (3H, s, H-14). ^{13}C -NMR (CD_3OD): δ 194.2(C-6) 160.2(C-8), 139.7(C-12), 132.8 (C-1), 132.0(C-10), 124.3(C-11), 123.0(C-7), 67.8(C-5), 65.2(C-4), 42.5(C-9), 39.0(C-3), 25.4(C-2), 15.7(C-15), 15.4(C-14), 10.7(C-13). From the comparison of these data with those reported in the literature [15–16], the substance was identified as zederone (Fig. 3). It is a sesquiterpene, was previously isolated from *C. amada* and *C. zedoaria* and has been reported for its analgesic, anti-inflammatory, anti-fungal and cytotoxic effects [12, 17–19–27].

Compound 2: Colorless oil; UV λ_{max} : nm 243, 280. ESI-MS (+) m/z : 231.0 $[\text{M} + \text{H}]^+$, 223.3 $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$. ^1H -NMR (CD_3OD): δ 7.16 (1H, s, H-12), 5.83 (1H, s, H-5), 5.21 (1H, dd, $J = 12$ Hz, 5 Hz, H-1), 3.73 (1H, d, $J = 16$ Hz, H-9a), 3.63 (1H, d, $J = 16$ Hz, H-9b), 2.45 (1H, ddd, $J = 15$ Hz, 11 Hz, 4 Hz, H-3a), 2.31 (1H, m, H-2a), 2.20 (1H, dddd, $J = 12$ Hz, 12 Hz, 12 Hz, 4 Hz, H-2b), 2.06 (3H, s, H-13), 1.92 (3H, s, H-14), 1.89 (1H, m, H-3b), 1.25 (3H, s, H-15). ^{13}C -NMR (CD_3OD): δ 191.8 (C-6), 158.6 (C-8), 147.6 (C-4), 140.0 (C-12), 136.1 (C-10), 133.3 (C-5), 132.0 (C-1), 124.6 (C-11), 123.1 (C-7), 42.4 (C-9), 41.4 (C-3), 27.3 (C-2), 19.3 (C-14), 15.9 (C-2), 9.9 (C-13). From the comparison of these data with those reported in the literature [20], the substance was identified as furanodienone (Fig. 3). It was isolated from *Lindera pulcherrima* (Nees.) Benth. ex hook. f [21], *Curcuma zedoaria* [19], *Curcuma amada* [12] and *Curcuma wenyujin* [22]. It is a furanosesquiterpenoid that exhibits anti-fungal [12], anti-inflammatory [19], anti-cancer [23], anti-bacterial and anti-oxidant activities [21].

Compound 3: Yellowish oil; UV λ_{max} : nm 275. ESI-MS (+) m/z : 383.3 $[\text{M} + \text{Na}]^+$, 361.3 $[\text{M} + \text{H}]^+$, 343.2 $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$. ^1H -NMR (CD_3OD): δ 6.99 (2H, dd, $J = 9, 2$ Hz, H-2'', -6''), 6.67 (2H, d, $J = 9$ Hz, H-3'', -5''), 6.52 (2H, s, H-2', -6'), 4.63 (1H, br b, $J = 12$ Hz, H-1), 4.21 (1H, m, H-3), 3.89 (1H, m, H-5), 3.85 (3H, s, 5'-OCH₃), 2.63 (2H, m, H-7), 1.82 (1H, m, H-2a), 1.78 (1H, m, H-6a), 1.73 (1H, m, H-2b), 1.69 (1H, m, H-4a), 1.68 (1H, m, H-6b), 1.53 (1H, m, H-4b). ^{13}C -NMR (CD_3OD): δ 156.3 (C-4''), 149.5 (C-5'), 146.4 (C-3'), 134.5 (C-4'), 134.44 (C-1'), 134.41 (C-1''), 130.4 (C-2'', -6''), 116.1 (C-3'', -5''), 108.0 (C-2'), 102.8 (C-6'), 75.2 (C-1), 72.6 (C-5), 65.6 (C-3), 56.6 (5'-OCH₃), 41.1 (C-2), 39.5 (C-4), 39.2 (C-6) 31.8 (C-7). From the comparison of these data with those reported in the literature [24], the substance was identified as 1,5-epoxy-3-hydroxy-1-(3,4-dihydroxy-5-methoxyphenyl)-7-(4-hydroxyphenyl) heptanes (Fig. 3). It was isolated from the rhizomes of *Zingiber officinale*, and their antioxidant properties were studied [24].

Compound 4: Viscous syrup; UV λ_{max} : nm 281. ESI-MS (+) m/z : 385.3 $[\text{M} + \text{Na}]^+$, 363.3 $[\text{M} + \text{H}]^+$, 345.2 $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$. ^1H -NMR (CD_3OD): δ 6.75 (1H, d, $J = 2$ Hz, H-2'), 6.68 (1H, d, $J = 8$ Hz, H-5'), 6.64 (1H, $J = 8$ Hz, H-

5''), 6.61 (1H, $J = 2$ Hz, H-2''), 6.60 (1H, dd, $J = 8, 2$ Hz, H-6'), 6.49 (1H, dd, $J = 8, 2$ Hz, H-6''), 3.80 (3H, s, 3'-OCH₃), 3.73 (2H, m, H-3, -5), 2.64–2.47 (4H, m, H-1a, -1b, -7a, -7b), 1.71–1.65 (4H, m, H-2a, -2b, -6a, -6b), 1.61 (2H, m, H-4a, -4b). ¹³C-NMR (CD₃OD): δ 148.8 (C-3'), 146.1 (C-3''), 145.4 (C-4'), 144.2 (C-4''), 135.24 (C-1' or C-1''), 135.22 (C-1' or C-1''), 121.8 (C-6'), 120.6 (C-6''), 116.5 (C-2''), 116.3 (C-5''), 116.1 (C-5'), 113.2 (C-2'), 70.94 (C-3 or C-5), 70.92 (C-3 or C-5), 56.4 (3'-OCH₃), 44.9 (C-4), 40.8 (C-2, -6), 32.3 (C-1), 32.1 (C-7). From the comparison of these data with those reported in the literature [25–26], the substance was identified as oil 3,5-dihydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl) heptanes (Fig. 3). It was isolated from the rhizomes of *Tacca chantrieri* [25] and *Curcumalonga* L. [26]. They are diarylheptanoids that exhibit cytotoxic [25] and anti-tumor [26] activities.

Compound 5: Colorless oil; UV λ_{\max} nm: 279. ESI-MS (+) m/z : 413.3 [M + Na]⁺, 391.3 [M + H]⁺, 373.3 [M + H-H₂O]⁺. ¹H-NMR (CD₃OD): δ 6.76 (1H, s, H-2''), 6.67 (1H, d, $J = 8$ Hz, H-5''), 6.21 (1H, dd, $J = 8, 2$ Hz, H-6'), 6.53 (2H, s, H-2', -6''), 4.63 (1H, br d, $J = 12$ Hz, H-1), 4.21 (1H, m, H-3), 3.83 (3H, s, 5'-OCH₃), 3.78 (3H, s, 3-OCH₃), 2.65 (2H, m, H-7), 1.84 (1H, m, H-2a), 1.79 (1H, m, H-6a), 1.74 (1H, m, H-2b), 1.69 (1H, m, H-4a), 1.68 (1H, m, H-6b), 1.53 (1H, m, H-4b). ¹³C-NMR (CD₃OD): δ 149.5 (C-5'), 148.8 (C-3''), 146.4 (C-3'), 145.4 (C-4''), 135.3 (C-1'), 135.2 (C-1''), 134.4 (C-4'), 121.9 (C-6''), 116.1 (C-5''), 113.4 (C-2''), 108.0 (C-2'), 102.7 (C-6'), 75.2 (C-1), 72.5 (C-5), 65.6 (C-3), 56.6 (5'-OCH₃), 56.3 (3'-OCH₃), 41.2 (C-2), 39.4 (C-4), 39.3 (C-6), 32.2 (C-7). From the comparison of these data with those reported in the literature [27], and there is no information about their biological activity. We isolated compounds **3**, **4** and **5** for the first time from *C. amada*. All the compounds showed inhibitory activity against mushroom tyrosinase.

Five compounds, namely, zederone, furanodienone, 1,5-epoxy-3-hydroxy-1-(3,4-dihydroxy-5-methoxyphenyl)-7-(4-hydroxyphenyl) heptanes, 3,5-dihydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl) heptanes and 1,5-epoxy-3-hydroxy-1-(3,4-dihydroxy-5-methoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl) heptanes, were isolated from fractions F3 and F6. The isolated compounds showed anti-tyrosinase activity in a concentration-dependent manner. Among the five compounds, compound **4** showed significantly stronger anti-tyrosinase activity than arbutin. There were no significant differences in the anti-tyrosinase effects between compound **5** and arbutin (Fig. 4).

The effects of the isolated compound on cellular viability were studied on B16F10 murine melanoma cells. The cells were treated with 50, 100, 200, and 400 μ M concentration of the compound for 48 h. The isolated compounds did not show cytotoxic effects up to 200 μ M, however, about fifty percent of cellular deaths were observed in all cases at 400 μ M concentration (data not shown). Thus, concentrations up to 200 μ M were used to assess their intracellular anti-tyrosinase and anti-melanogenic effects.

To determine the anti-melanogenic and anti-tyrosinase activities of the isolated compounds, their effects on melanin content and tyrosinase activity were evaluated in B16F10 melanoma cells. As shown in Table 1, our isolated compounds dose-dependently inhibited intracellular melanin content and tyrosinase activity. Compound **4** was significantly stronger than the positive control drug arbutin in both melanin

and tyrosinase inhibitory effects. No significant difference was observed between compound 5 and arbutin.

Table 1

IC₅₀ of the isolated compounds against intracellular tyrosinase and melanin content in B16F10 murine melanoma cells

Compound	IC ₅₀ (μM) for Cellular Tyrosinase Inhibition	IC ₅₀ (μM) for Melanin Content Inhibition
Compound 1	174.5 ± 5.1 ^a	202.2 ± 5.2 ^a
Compound 2	169.7 ± 6.7 ^a	213.4 ± 6.6 ^a
Compound 3	188.3 ± 6.2 ^b	199.3 ± 7.7 ^a
Compound 4	42.4 ± 1.3 ^c	54.7 ± 1.6 ^b
Compound 5	98.7 ± 3.8 ^d	145.6 ± 5.4 ^c
Arbutin	93.1 ± 4.1 ^d	153 ± 6.9 ^c
Different letters in the same column indicate the existence of significant difference statistically. Values represented as mean ± SEM.		

As cellular tyrosinase enhances melanin production, reduction of tyrosinase activity is an efficient strategy for the development of anti-melanogenic agents. For this, we evaluated the inhibitory properties of intracellular tyrosinase activity and melanogenesis in B16F10 melanoma cells. Similar to the findings of mushroom tyrosinase inhibitory activity, the isolated compounds inhibit intracellular tyrosinase activity and melanogenesis in a dose-dependent manner. The anti-tyrosinase effects of the isolated compounds resulted in their anti-melanogenic properties. The order of anti-tyrosinase and anti-melanogenic activity of these compounds was compound **4** > **arbutin** > **5** > **2** > **3** > **1**. The calculated IC₅₀ value of compound **4** was significantly lower than that of arbutin. The effectiveness of compound **4** was 1.9- to 5-fold higher than those of the other four compounds. Compound **5** also showed strong anti-tyrosinase activity that was comparable with that of arbutin. Our results indicated that compounds **4** and **5** could be used as potential natural tyrosinase inhibitors and skin-whitening cosmetics. Oxidative stress has been proposed to be involved in the underlying mechanism of the overproduction of melanin [28]. Therefore, the antioxidant role has been investigated in a wide range of skin disorders, including photocarcinogenesis or melanoma [9]. We and others previously reported the anti-oxidant properties of *C. amada* ([13, 29] that might be responsible for the anti-melanogenic effects of the isolated compounds. In this study, we detected inhibitory effects of the isolated compounds on intracellular melanin synthesis and tyrosinase activity induced by α-MSH. Thus, our isolated compounds can be employed as agents in functional cosmetics to develop effective skin-whitening treatments.

Conclusion

We strongly suggest that *C. amada* can play an important role as an effective tyrosinase inhibitor. *C. amada* and its bioactive compounds could be used in the cosmetic industry as natural whitening agents, the food industry as anti-browning agents and the medical field for the treatment of hyperpigmentation. Nevertheless, further studies are needed to investigate the anti-melanogenic effects of the isolated compounds in animal models.

Materials And Methods

Chemicals

Tyrosinase from mushroom and arbutin were purchased from Sigma-aldrich chemical Co. (St. Louis, MO, USA). L- Tyrosine was from Wako pure chemical industries Ltd. (Osaka, Japan). Methanol (MeOH), ethyl acetate (EtOAc) and *n*-hexane were purchased from Nacalai Tesque (Kyoto, Japan). Silica gel (63–200 µm, Kanto Chemical Co. Tokyo, Japan) and MeOH-*d*₄(CD₃OD, Merck KGaA, Germany) were purchased.

Plant material preparation

Four different species of turmeric namely *C. xanthorrhiza*, *C. aromatica*, *C. amada*, and *C. zedoaria* were cultivated in a field of gray soil (coarse sand 3.6%, fine sand 30.9%, silt 24.3%, clay 32.8%, pH 7.4, NO₃-N 0.07%, NH₄-N 0.08%, P 4.6 ng/g, K 42.9 ng/g) at the Subtropical Field Science Center, University of the Ryukyus, Okinawa, Japan. The average monthly temperature, humidity, and precipitation during the cultivation period were 17–29°C, 61–83% and 22–369 mm, respectively. Common agronomic practices including fertilizer and irrigation were provided. Rhizomes were harvested when all the shoots of the species withered completely. The rhizomes were washed, sliced and dried in a hot air oven at 50°C for 72 h.

Extraction of Samples

The extractions were carried out by dissolving the different turmeric powder (300 g) into MeOH (3L) at room temperature (25°C) and at atmospheric pressure and kept for 2 days with continuous magnetic stirring to prevent oxidation by air and shielding from sunlight. The solvent-soluble compounds were filtered using a filter paper (No. 2, Advantec, Tokyo Roshi Kaisha Ltd., Tokyo, Japan). Fresh solvents (MeOH) were added into the used plant material and the process was repeated three times. The filtered solutions containing plant compounds were dried by rotary evaporator under reduced pressure at 40°C. The yield of all extracts was kept in refrigerator at 4°C for experimental analyses.

Tyrosinase Inhibition Assay

Tyrosinase inhibition activity was determined according to the previous method [30], measuring the concentration of DOPA chrome produced by the action of tyrosinase enzyme on tyrosine substrate. Briefly, the test sample was dissolved in 80% MeOH to obtain different concentrations (25, 50, and 100 µg/mL). The 96-well plate was set up in the following order: 120 µL of phosphate buffer (20 mM, pH 6.8),

20 μ L of the sample, and 20 μ L of mushroom tyrosinase (500 U/mL in 20 mM phosphate buffer). After incubation for 15 min at 25°C, the reaction was initiated by adding 20 μ L of 0.85 mM L-tyrosine solution and was then incubated for 10 min at 25°C. Tyrosinase activity was determined by measuring the absorbance at 470 nm using a microplate reader (Biotek Powerwave XS2 spectrophotometer). Arbutin was used as a positive control, whereas 80% MeOH was used as a negative control. The percentage of tyrosinase inhibition was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = [1 - (S - B)/C] \times 100$$

where C is the absorbance of the negative control, B is the absorbance of the blank, and S is the absorbance of the test sample.

Isolation of bioactive compounds from the crude extract of Curcuma amada

Considering the results of the four turmeric extracts, *C. amada* showed significantly higher anti-tyrosinase activity than the others. Therefore, bioassay-guided purification of active compounds from crude extract of *C. amada* was performed. To identify the anti-tyrosinase compounds, fractionation from the crude extract of *C. amada* was carried out as described in Fig. 1.

The crude extract was diluted with distilled water and then extracted with *n*-hexane, followed by EtOAc. Equal volumes of each solvent and crude extract solution were then mixed by shaking for 3 min in a separation funnel. All fractions were concentrated to dryness by a rotary evaporator at 40°C. The anti-tyrosinase activities of these three fractions were determined according to the above procedure.

As the EtOAc fraction showed the highest anti-tyrosinase activity, it was selected for the isolation and purification of the bioactive compound. The active EtOAc fraction was evaporated to dryness and subjected to chromatography on a silica gel (75 g) column (30 cm x 3 cm). Elution was carried out using *n*-hexane and EtOAc with increasing amounts of EtOAc [100:0 (F1), 80:20 (F2), 60:40 (F3), 40:60 (F4), 20:80 (F5) and 0:100 (F6)]. The anti-tyrosinase activity of these six fractions was carried out according to the above procedure, and most activity was found in F3 and F6. Fractions F3 and F6 were purified by C₁₈ reversed-phase HPLC (COSMOSIL 5C₁₈-AR-II; Nacalai Tesque, Inc., Kyoto, Japan) equipped with water and acetonitrile as the mobile phase with a flow rate of 2.5 mL min⁻¹, detected at 280 nm. Three peaks from F3 eluted at 9.55, 13.66 and 16.47 min, and four peaks from F6 eluted at 11.56, 12.20, 12.75 and 15.03 min as colorless white substances, of which inhibitory activity was detected in five peak fractions eluted at 9.55 and 16.47min from F3 and 11.56, 12.75 and 15.03 min from F6 (Fig. 2). The isolated compounds (approximately 10 mg) were dissolved in MeOH-*d*₄ and then subjected to spectral analysis. Nuclear magnetic resonance (NMR) spectra were recorded on BRUKER NMR spectrometers (500 MHz for ¹H and 125 MHz for ¹³C) at room temperature. Chemical shifts (δ) were recorded as parts per million (ppm) relative to tetramethylsilane (TMS) as the internal standard. Mass spectrometry experiments were carried out on a Waters Mass Spectrometer using an electrospray ionization (ESI - MS) probe under the following instrumental conditions: Column: COSMOSIL 5C18-AR-II, (2x150) mm. Solvent A: Water (0.1% formic acid), Solvent B: acetonitrile, flow rate: 4 mL/min, injection volume: 100 μ L, run time: 35 min, time program for F3: 75% B (0 min) \rightarrow 75% B (20 min) \rightarrow 100% B (20.1 min) \rightarrow 100% B (27 min) \rightarrow 75% B (27.1

min)→75% B (35 min). Time program for F6: 45% B (0 min)→45% B (14 min)→100% B (14.1 min)→100% B (20 min)→45% B (20.1 min)→45% B (25 min). Pump mode: Binary gradient. Oven details: CTO-20AC, temperature 40°C. MS ionization mode: ES (+), capillary voltage: 4.0 kV, cone voltage: 20 V, source temp.: 120°C, desolvation temp.: 350°C, cone gas flow: 100 L/h, desolvation gas flow: 800 L/h.

Anti-tyrosinase activity of the isolated compounds

Isolated compounds were dissolved in MeOH at concentrations of 5, 10, 30, 50, 100 and 200 µM for each compound. Anti-tyrosinase activity was determined using the procedure described before.

Intracellular inhibition of tyrosinase and melanogenesis by the isolated compounds

Cell Culture

B16F10 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin (10,000 U/mL)/streptomycin (100 µg/mL) at 37°C in a humidified atmosphere containing 5% CO₂.

Cell Viability

B16F10 cells were plated at a density of 5×10^4 cells/well in a 96-well plate. After 24 h, cells were exposed to various concentrations of the isolated compound and incubated for additional 48 h at 37°C. Following incubation, cell viability was determined by the MTS assay. Twenty microliters of MTS solution was added and incubated for 60 min. After incubation, the absorbance of the cells was determined at 490 nm using a microplate reader (Benchmark Plus).

Anti-tyrosinase and anti-melanogenic activities of the isolated compounds

The determination of cellular melanin content and tyrosinase activity assays were conducted as previously described [31], with slight modifications. B16F10 melanoma cells were plated at a density of 5×10^4 cells/well in a 96-well plate. After 24 h, cells were exposed to various concentrations of the isolated compound or arbutin. After 1 h, 100 nM α-melanocyte stimulating hormone (α-MSH) was added, and the cells were incubated for an additional 48 h at 37°C. For the anti-tyrosinase activity study, the cells were then washed with ice-cold phosphate buffer and lysed with phosphate buffer (pH 6.8) containing 1% Triton-X (90 µL/well). The plates were frozen at -80°C for 30 min. After thawing and mixing, 10 µL of 1% L-DOPA was added to each well. Following incubation at 37°C for 2 h, the absorbance was measured at 490 nm. For the melanin content assay, the cells were washed twice with phosphate buffer and then dissolved in 100 µL of NaOH (1 N) containing 10% DMSO. Samples were incubated at 80°C for 1 h and mixed to solubilize melanin. The optical density of the mixed homogenate was measured at 490 nm.

Statistical analysis

Results are expressed as the mean ± SEM. Statistical differences between two means were evaluated by the Student's *t*-test. Multiple comparisons were performed using one-way analysis of variance followed by Bonferroni test. Differences were considered significant at $P < 0.05$.

Declarations

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Conflicts of interest

The authors declare no conflict of interest.

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Figures

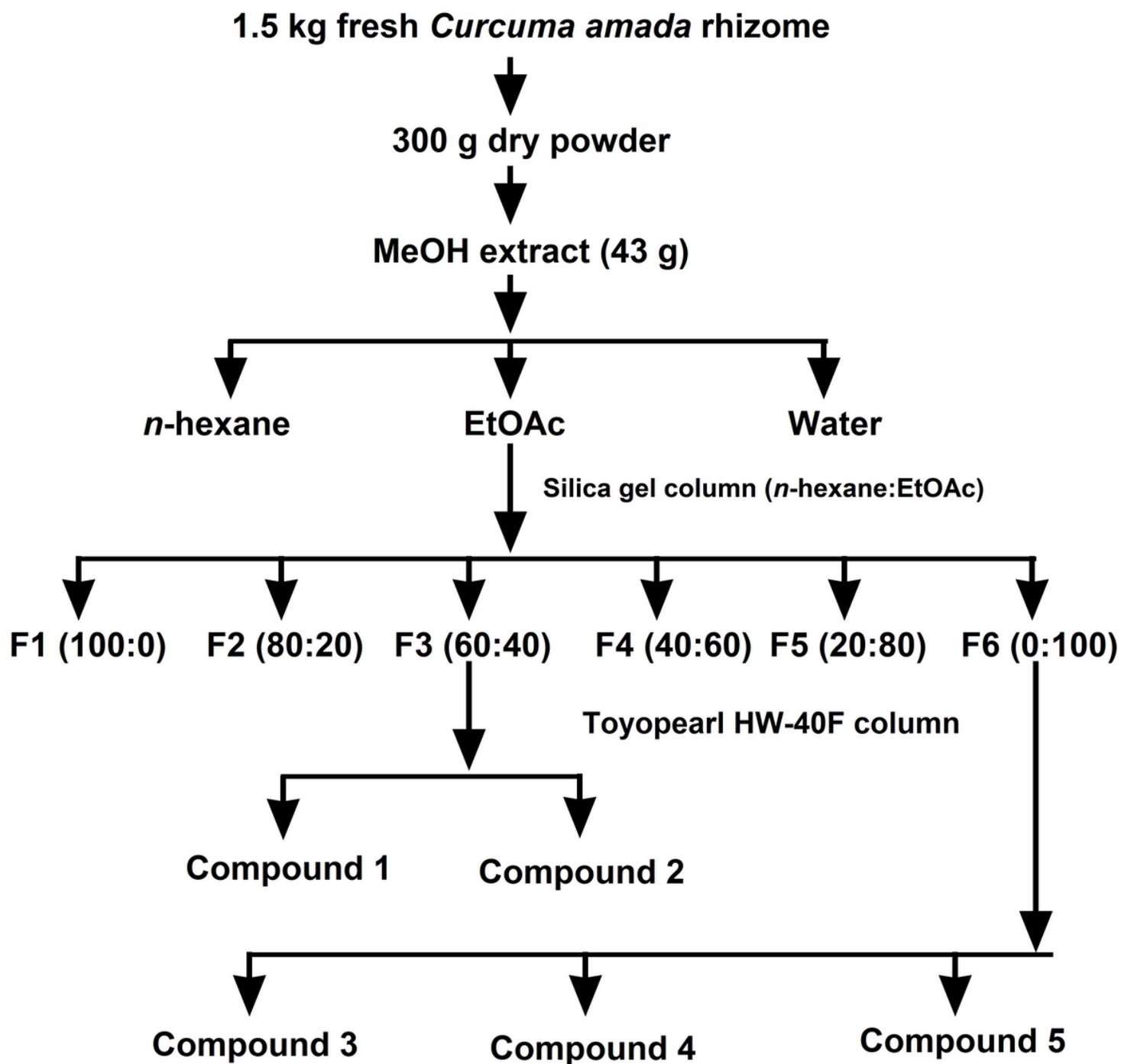


Figure 1

Flow chart for the bioassay-guided isolation and purification of active compounds from *C. amada*.

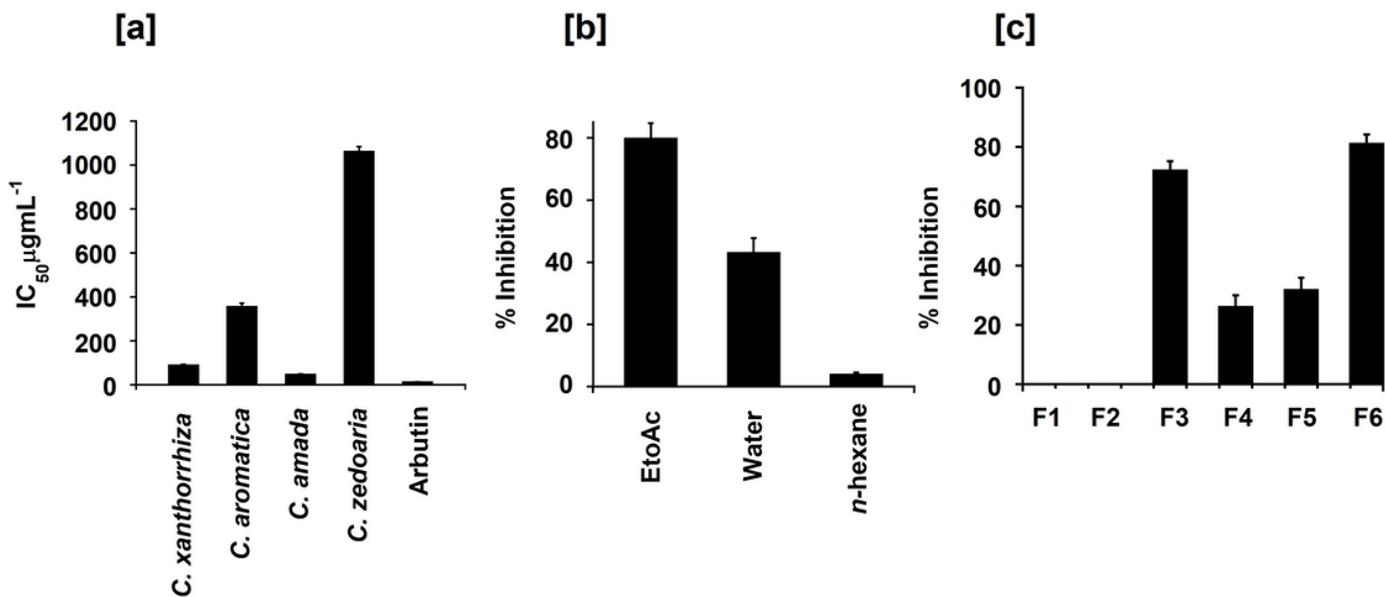


Figure 2

Anti-tyrosinase activities of different species of turmeric (3a), different solvent extracts (3b) and six fractions (3c) of *C. amada*. The results are expressed as the mean \pm SEM of three independent experiments in triplicate. * $P < 0.05$ vs. other fractions.

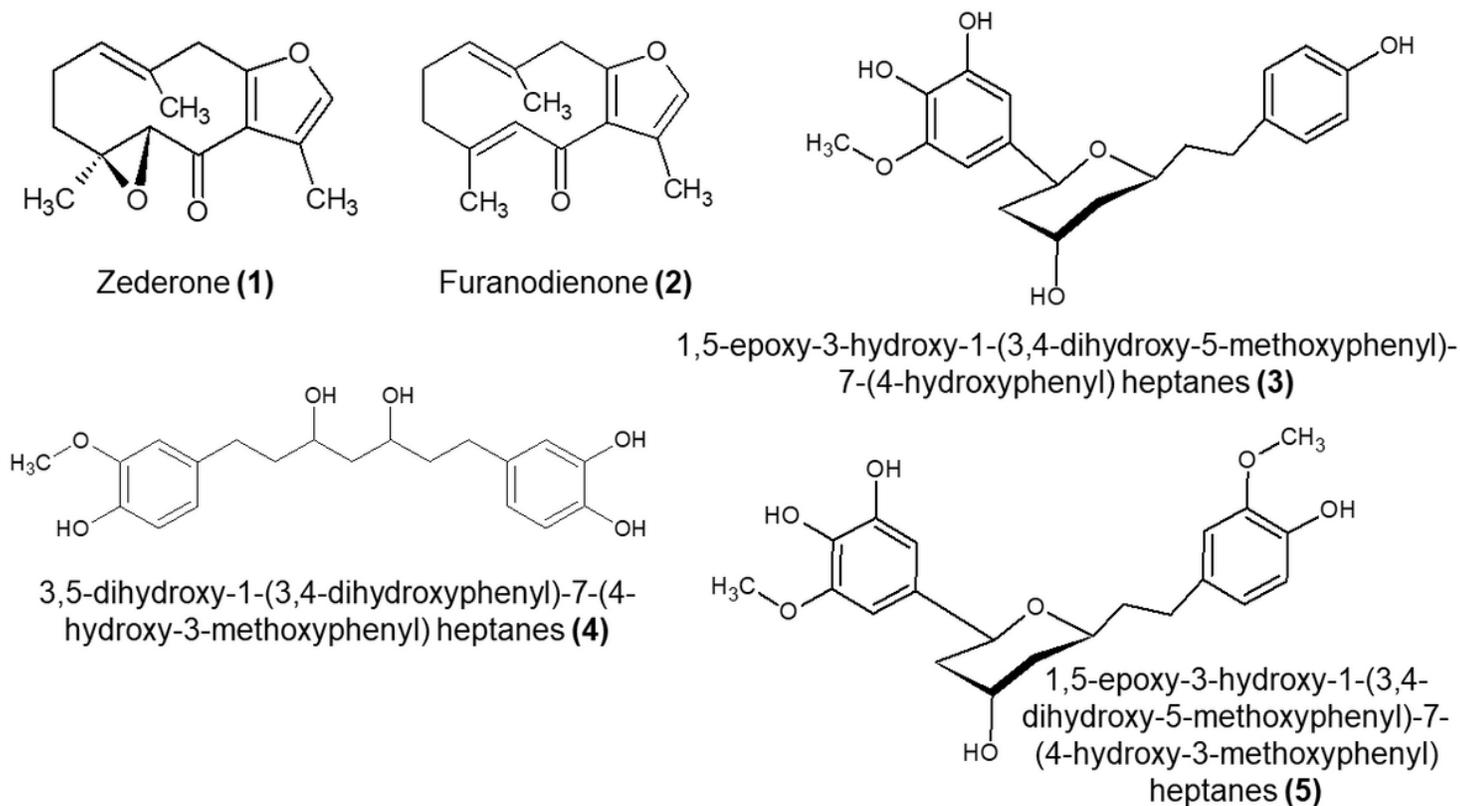


Figure 3

The chemical structures of the five compounds isolated from *C. amada* were identified according to their ¹H NMR and ¹³C NMR spectra: zederone (1), furanodienone (2), 1,5-epoxy-3-hydroxy-1-(3,4-dihydroxy-5-methoxyphenyl)-7-(4-hydroxyphenyl) heptanes (3), 3,5-dihydroxy-1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl) heptanes (4) and 1,5-epoxy-3-hydroxy-1-(3,4-dihydroxy-5-methoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl) heptanes (5).

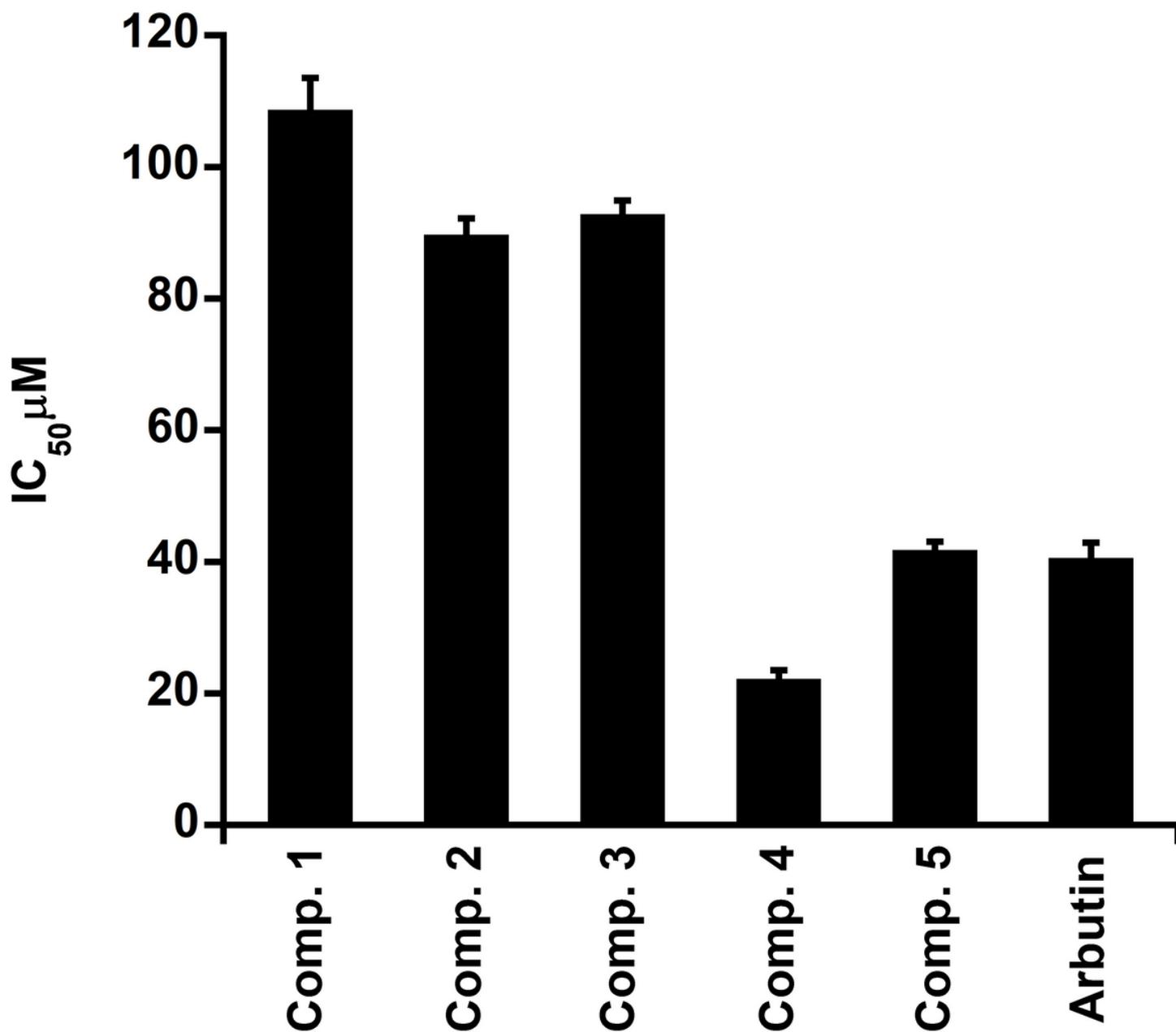


Figure 4

Effects of the isolated compounds from *C. amada* on mushroom anti-tyrosinase activity. The results are expressed as the mean ± SEM of the IC₅₀ of three independent experiments in triplicate. *P<0.05 vs. arbutin.

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