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ORIGINAL PAPER

Callicarpa longissima extract, carnosol-rich, potently inhibits melanogenesis in B16F10 melanoma cells

Minori Yamahara[1,2] · Koji Sugimura[3] · Ayako Kumagai[4] · Hiroyuki Fuchino[5] · Azusa Kuroi[2,4] · Mai Kagawa[4] · Yumi Itoh[4] · Hidehisa Kawahara[6] · Yasuo Nagaoka[6] · Osamu Iida[3,5] · Nobuo Kawahara[3,5] · Hiroshi Takemori[4] · Hideto Watanabe[1]

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Abstract Cosmetic industries focus on developing materials and resources that regulate skin pigmentation. Melanin, the major pigment in human skin, protects the skin against damage from ultraviolet light. An ethanolic extract of the leaves of *Callicarpa longissima* inhibits melanin production in B16F10 mouse melanoma cells by suppressing microphthalmia-associated transcription factor (MITF) gene expression. Following purification and analysis using liquid chromatography–mass spectrometry (LC–MS), NMR, and biochemical assays, carnosol was determined to be responsible for the major inhibitory effect of the *C. longissima* extract on melanin production. Carnosol is an oxidative product of carnosic acid, whose presence in the extract was also confirmed by an authentic reference. The carnosol and carnosic acid content in the extract was approximately 16 % (w/w). These results suggest that *C. longissima* is a novel, useful, and attractive source of skin-whitening agents.

Keywords Melanogenesis · MITF · Carnosol · Carnosic acid · B16F10

✉ Hiroshi Takemori
mukuharu22@yahoo.co.jp

1 Institute for Molecular Science of Medicine, Aichi Medical University, 1-1, Yazakokarimata, Nagakute, Aichi 480-1195, Japan

2 Department of Research and Development, Momotani-juntenkan Co. Ltd, 2-4-30, Ichioka, Minato-ku, Osaka 552-0012, Japan

3 Tanegashima Division, Research Center for Medicinal Plant Resources, Japan National Institute of Biomedical Innovation, Health and Nutrition, 17007-2, Matsubarayama, Noma, Nakatane-cho, Kumage-gun, Kagoshima 891-3604, Japan

4 Cell Signaling and Metabolic Disease Laboratory, Department of Fundamental Technological Research, Japan National Institute of Biomedical Innovation, Health and Nutrition, 7-6-8, Asagi-Saito, Ibaraki, Osaka 567-0085, Japan

5 Tsukuba Division, Research Center for Medicinal Plant Resources, Japan National Institute of Biomedical Innovation, Health and Nutrition, 1-2, Hachimandai, Tsukuba, Ibaraki 305-0843, Japan

6 Department of Life Science and Biotechnology, Kansai University, Suita, Osaka 564-8680, Japan

Introduction

Aged human skin is characterized by hyperpigmentation, in addition to wrinkles and decreased elasticity, leading to frequent reliance on cosmetics [1]. Hyperpigmentation results from an increase in the number or activity of melanocytes [2]. Melanin synthesis is regulated by tyrosinase (TYR) (EC 1.14.18.1), which catalyzes hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine and its subsequent oxidation to dopaquinone. Dopaquinone is converted through dopachrome into an indole derivative via autoxidation, and the conjugation of dopaquinone with this indole derivative produces melanin [3].

TYR activity is regulated by a multi-step process involving several molecules. Ultraviolet radiation stimulates keratinocytes to synthesize and secrete α-melanocyte-stimulating hormone [4], a paracrine hormone that binds to the melanocortin 1 receptor on the melanocyte membrane, increases cAMP levels, and activates protein kinase A (PKA). Active PKA phosphorylates the transcription factor cAMP response element-binding protein (CREB), up-regulating transcription of microphthalmia-associated transcription factor (MITF) [5, 6]. In turn, MITF binds to the *Tyr* promoter region and up-regulates its expression.

Both natural and synthetic sources contain melanin synthesis inhibitors. Only a few of these have been approved under strict governmental regulations for use in skin-whitening products. These include linoleic acid [7], hinokitiol [8], kojic acid [9], hydroquinone [10], catechols [11], and salicylate [12]. In a previous study, we identified the ethanolic extract of *Callicarpa longissima* [13], which potently inhibits melanin synthesis in B16F10 mouse melanoma cells by suppressing MITF expression, as a new resource for skin-whitening compounds. Further analysis suggested that carnosol and carnosic acid in this extract are responsible for inhibiting melanin synthesis. The present study aimed to investigate this relationship.

Materials and methods

Plant extracts and chemicals

Plant materials from fields of the Tanegashima and Tsukuba Divisions of the Research Center for Medicinal Plant Resources, Japanese National Institute of Biomedical Innovation, were dried (LC-234 Dryer, ESPEC, Tokyo, Japan) at 50 °C for 2–3 days and ground into powder. Five grams of the powder was soaked in 20 mL of ethanol for 2 days and the extract was then passed through 2 g of activated charcoal (Wako Pure Chemicals Co., Ltd., Kyoto, Japan) to exclude chlorophyll and related compounds. The ethanol was evaporated and the extracts were resuspended in 10 mg/mL ethanol. Authentic carnosol and carnosic acid were obtained from Wako. Antibody reagents of the following were obtained: anti-tyrosinase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-MITF (Thermo Fisher Scientific Inc. Waltham, MA, USA); and β-actin, pp38, pERK1/2, pMEK1/2, pp90Rsk, and pCREB (Cell Signaling Technology, Danvers, MA, USA). Antigen–antibody interactions were visualized using ECL chemiluminescence reagent (GE Healthcare, Uppsala, Sweden) followed by detection with LAS4000 (Fuji Film, Tokyo, Japan), or use of a chromogenic reaction with immunostain horseradish peroxidase (HRP) (ATTO Co., Ltd., Tokyo, Japan).

Cell culture and reporter assay

Mouse B16F10 melanoma cells from the American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, 5.5 mM D-glucose), supplemented with 10 % fetal bovine serum and antibiotics. The cells were incubated in a humidified atmosphere with 5 % CO_2 at 37 °C and were transferred every 2 days via trypsinization to new culture dishes. Cells plated in 6-well dishes were stimulated with forskolin (Fsk, 10–15 μM) and cultured for 3 or 48 h for MITF or TYR/melanin assays, respectively.

To perform reporter analyses, the 2-kb promoter region of the mouse *Tyr* gene was amplified by PCR using two primers: 5′-TCT ATC GAT AGG TAC CGG GTA GAC AAT AGT CAA ACA G-3′ and 5′-CTT AGA TCG CAG ATC TTC TGC ACC AAT AGG TTA ATG AGT G-3′. After digestion with *Acc*65I/*Bgl*II, the promoter DNA fragment was ligated into the corresponding site of the pGL4 firefly luciferase vector and then identified as pGL4-mouse *tyrosinase*.

To prepare a *Mitf* overexpression vector, the mouse *Mitf* cDNA fragment was amplified by PCR using a cDNA pool of B16F10 melanoma cells and two primers: 5′-TTT AGA TCT ATG CTA GAA TAC AGT CAC TAC CA-3′ and 5′-AAA GCG GCC GCT CGC TAA CAC GCA TGC TCC GT-3′. After digestion with *Bgl*II and *Not*I, the amplified cDNA fragment was ligated into the corresponding site of the pTarget vector (Promega, Madison, WI, USA). B16F10 melanoma cells plated onto a 24-well plate were cotransfected with pGL4 mouse *tyrosinase* (0.2 μg/well) with the internal reporter pRL-TK (0.03 μg), in the presence or absence of the *Mitf* expression vector (pTarget-mouse *Mitf*, 0.1 μg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h, the cells were exposed to Fsk (15 μM) and cultured further for 24 h. Reporter activity was monitored using the Dual Luciferase Reporter Assay Kit (Promega). The quantitative PCR analyses, including primer sets, have been described in [6].

Assay for melanin content and tyrosinase activity

Melanin measurement was performed as in [14]. Briefly, the cells were washed twice with phosphate-buffered saline and recovered in 2-mL collection tubes, followed by centrifugation at 8000 rpm for 1.5 min. Each cell pellet was suspended in 300 μL of 1 M NaOH and then lysed by incubation at 45 °C for 2 h. Melanin was extracted with a 2:1 chloroform–methanol mixture and measured with a spectrophotometer (Model 680; Bio-Rad, Hercules, CA, USA) at 405 nm. The protein concentration of the cell pellets was determined using Bradford reagent (Bio-Rad) and used for normalization of the melanin content.

TYR activity was measured according to the method described in [15]. Cells in 6-well plates were stimulated with 15 μM Fsk for 48 h and collected in 2-mL tubes. After recovery as cell pellets, the cells were lysed in 500 μL 0.5 % sodium deoxycholate supplemented with 200 μM PMSF at 4 °C for 30 min; thereafter, 80 μL of cell lysates were incubated with 400 μL assay buffer (2×) (100 mM sodium phosphate buffer, pH 7.4, 4 % *N*,*N*′-dimethylformamide), 200 μL of 5 mM L-DOPA, and 200 μL of 20.7 mM 3-methyl-2-benzothiazoline hydrazone at

37 °C for 30 min. TYR activity was monitored by absorbance (505 nm) and normalized by amount of protein.

Purification of carnosol and its analysis

Forty grams of dried powder made from the leaves of *C. longissima* were soaked in 400 mL of ethanol and extracted three times. The resulting extract was concentrated to 100 mL by evaporation and passed through 40 g of activated charcoal. To remove water-soluble compounds, 100 mL of hexane was added to the flow-through fraction and the viscous solution (approximately 5 mL) was discarded. Ethanol and hexane were evaporated and the extract was dissolved with chloroform and applied onto a silica gel column (100 mL; Nacalai Co. Ltd., Kyoto, Japan). Melanin-suppressing substances were eluted using a 9:1 chloroform:methanol solvent. The eluted materials were first suspended in 20 % acetonitrile–water and applied onto a C-18 column (4.6 mm × 50 mm; Cosmosil MS-II, Nacalai, San Diego, CA, USA), then suspended in 50 % acetonitrile–water of 90 % or greater purity, recovered in ethyl acetate, and analyzed by LC–MS (QSTAR, AB Sciex, Framingham, MA, USA) and ^{13}C-NMR (ECA-500, JEOL, Tokyo, Japan). The substance was extracted as carnosol.

^{1}H-NMR (CD_3OD) δ: 0.858, 0.864 (each 3H, s, H-19,18), 1.18, 1.19 (each 3H, d, $J = 6.3$ Hz, H-16,17), 1.29 (1H, ddd, $J = 16.5$, 16.5, 4.1 Hz, H-3ax), 1.49 (1H, brd, $J = 16.5$, H-3eq), 1.58 (1H, m, H-2eq), 1.68 (1H, dd, $J = 12.4$, 6.9 Hz, H-5), 1.83 (1H, m, H-6ax), 1.90 (1H, m, H-2ax), 2.18 (1H, m, H-6eq), 2.56 (1H, ddd, $J = 14.4$, 14.4, 4.6 Hz, H-1eq), 2.79 (1H, d, $J = 14.4$ Hz, H-1ax), 3.23 (1H, m, H-15), 5.41 (1H, dd, $J = 4.9$, 2.1 Hz, H-7), 6.68 (1H, s, H-14).

^{13}C-NMR (CD_3OD) δ: 30.1 (C-1), 20.1 (C-2), 42.2 (C-3), 35.5 (C-4), 47.0 (C-5), 30.8 (C-6), 79.7 (C-7), 133.3 (C-8), 122.9 (C-9), 49.5 (C-10), 144.2 (C-11), 144.7 (C-12), 136.0 (C-13), 112.5 (C-14), 27.9 (C-15), 23.21 (C-16), 23.24 (C-17), 32.1 (C-18), 20.1 (C-19), 179.4 (C-20).

Results

Screening of melanogenesis modulators

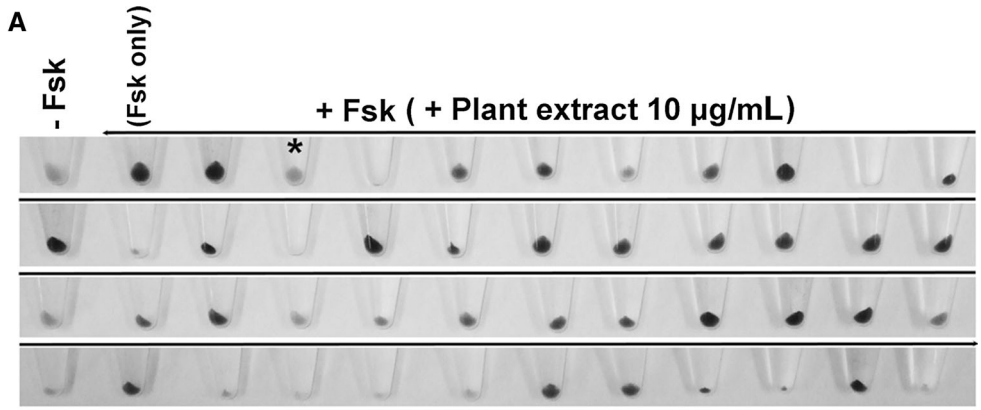
To identify plant extracts that modulate melanogenesis, we tested the leaf, stem, bark, and fruit of over 200 plants for inhibitory effects on melanin synthesis. The powdered plant materials were soaked in ethanol for 2 days. The extracts were passed through activated charcoal columns to remove chlorophyll and related compounds and concentrated to 10 mg/mL. B16F10 melanoma cells were treated with the extracts (10 μg/mL) in the presence of Fsk (10 μM), which increases cytoplasmic cAMP levels. After 2 days, our gross visual observations identified 10 candidates with inhibitory effects on melanin synthesis (Fig. 1a). Further examination of cells treated with the extracts at 1 μg/mL identified three extracts showing significant inhibitory effects on melanin synthesis (Fig. 1b), of which the extract from the leaves of *C. longissima* showed the strongest effect. Quantification of melanin content (Fig. 1c) and total TYR activity (Fig. 1d) showed significant inhibition of melanin synthesis by *C. longissima* extract at the lowest concentration of 1 μg/mL and in a dose-dependent manner. Therefore, subsequent experiments focused on this extract.

C. longissima extract inhibits the induction of MITF protein

Next, we investigated the effects of the *C. longissima* extract on protein levels of MITF and TYR. When we treated B16F10 cells with the *C. longissima* extract in the presence of Fsk, Western blots revealed that levels of MITF (Fig. 2a) and TYR (Fig. 2b) decreased in a dose-dependent manner. Time-course analysis revealed an increase in MITF protein level 180 min after Fsk stimulation, which was inhibited by co-treatment with the *C. longissima* extract (Fig. 2c). Although we similarly examined phosphorylation levels of several signal transduction molecules (p38, ERK, MEK, RSK, and CREB) known to affect MITF expression, we could not identify any clues to the mechanisms by which the *C. longissima* extract inhibited the MITF expression.

Overexpression of MITF rescues *C. longissima* extract-dependent suppression of the *Tyr* promoter activity

To investigate whether the reduced level of MITF protein in *C. longissima* extract-treated cells was the result of down-regulation of MITF gene expression, we performed quantitative PCR analysis of *Mitf* mRNA (Fig. 3a). Treatment with the *C. longissima* extract reduced *Mitf* and *Tyr* mRNA levels in unstimulated and Fsk-stimulated cells after 3 and 48 h, respectively (Fig. 3a, b). To investigate the importance of MITF levels on *Tyr* gene expression, we performed a promoter assay using the mouse *Tyr* promoter linked with the luciferase gene. Fsk stimulation up-regulated *Tyr* promoter activity four-fold, and treatment with the extract down-regulated *Tyr* promoter activity to approximately 30 % in both Fsk-stimulated and unstimulated cells (Fig. 3c). Overexpression of MITF in the cells abolished the inhibitory effect of the extract (Fig. 3d). Taken together, these results indicate that the *C. longissima* extract initially down-regulates MITF gene transcription



B

	Plant extract	portion	visual evaluation (dose) (1 μg/mL)	(10 μg/mL)
*	*Callicarpa longissima*	leaf	++	++
	Juglans mandshurica var. *sachalinensis*	fruit	+	++
	Terminalia chebula	leaf	−	+
	Piper betle	leaf	−	+
	Andrographis paniculata	stem	−	+
	Alpinia speciosa	stem	+	++
	Alpinia intermedia	stem	+	++
	Pimenta racemosa	bark	−	+
	Laurus nobilis	bark	−	+
	Murraya paniculata	bark	−	+

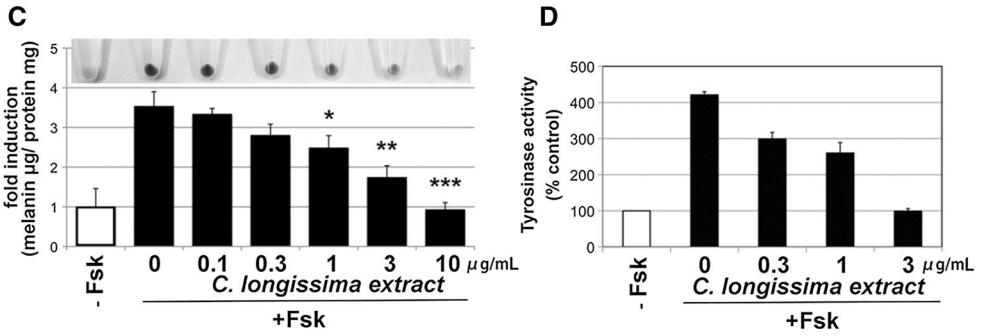


Fig. 1 Screening of plant extracts inhibiting melanogenesis. **a** Melanin production in B16F10 melanoma cells was induced by 10 μM of Fsk in the presence of a variety of plant extracts (10 μg/mL). After 48 h incubation, cells were collected into centrifuge tubes and melanin contents were judged by visual evaluation. *Asterisk* indicates cells treated with the *C. longissima* extract. **b** Positive extracts were further evaluated at 1 μg/mL. ++ indicates more than 75 % inhibition; + indicates 50 % inhibition by visual evaluation. **c** Melanin content was examined after normalization by protein concentration ($n = 3$, means and SD are indicated), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with Fsk only. **d** Total tyrosinase enzyme activity in the cells (**c**) was measured

and that a decrease in MITF leads to downregulation of TYR gene transcription, diminishing TYR protein levels and enzymatic activity.

Carnosol in *C. longissima* extract is the compound responsible for suppression of melanogenesis

To identify the functional compounds in the *C. longissima* extract, we fractionated the extract, evaluated the inhibitory effects of the fractions on pigmentation, purified a candidate to 90 % homogeneity, and performed LC–MS and ^{13}C-NMR analyses (see "Materials and methods"), resulting in carnosol as a candidate (Fig. 4a). Carnosol is an oxidative product of carnosic acid, abundant in some herbs including rosemary, whose extracts are known to suppress melanogenesis [16]. The presence of a substantial amount of carnosic acid in the *C. longissima* extract was also confirmed by an authentic standard. The total content of carnosol and carnosic acid in the *C. longissima* extract was approximately 16 % (w/w).

Compared with carnosol or carnosic acid alone, the *C. longissima* extract more strongly inhibited melanogenesis (Fig. 4b, c), suggesting that the extract exerts synergistic activity of carnosol and carnosic acid and possibly other

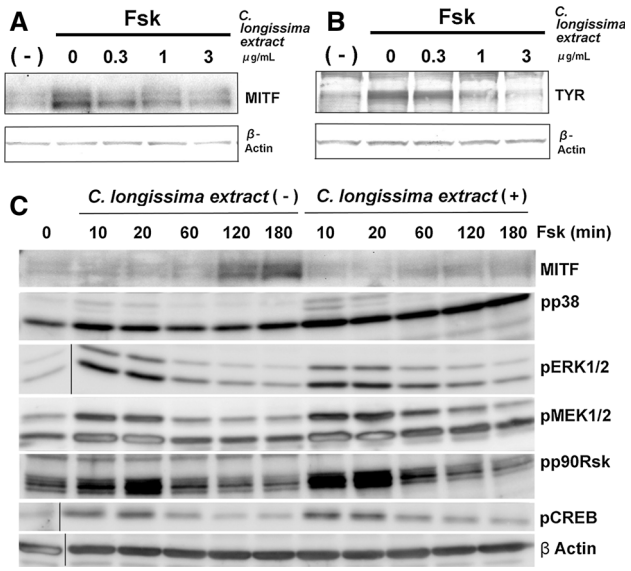


Fig. 2 MITF protein level is suppressed by *C. longissima* extract. **a** Western blot analysis of MITF protein in B16F10 melanoma cells treated with Fsk (15 μM) for 3 h. **b** Tyrosinase (TYR) protein levels were evaluated in cells incubated with Fsk for 48 h. **c** Time-dependent induction of MITF protein and phosphorylation status of signaling molecules were evaluated. B16F10 melanoma cells were incubated with the *C. longissima* extract (3 μg/mL) and Fsk (15 μM)

agents. Such synergism may also be helpful in averting cell toxicity compared with carnosol or carnosic acid alone (Fig. 4d).

Discussion

An ethanol extract of the leaves of *C. longissima* strongly inhibited melanin production in mouse B16F10 melanoma cells. The substances in the extract responsible for melanogenesis inhibition were carnosol and carnosic acid.

Both carnosol and carnosic acid are gaining attention for their antioxidant properties [17–19]. Their anti-melanogenic potencies have also been reported as suppressors of TYR expression in B16 melanoma cells [16]. Carnosol is formed from carnosic acid by autoxidation through its intermediate carnosic acid quinone [20]. Although carnosic 8-lactone and rosmanol are also produced via the same oxidative reaction, their contributions to the suppression of melanogenesis are as yet unknown, probably owing to their unstable nature.

Cellular targets of carnosol and carnosic acid have been identified. They include CREB-binding protein/E1A

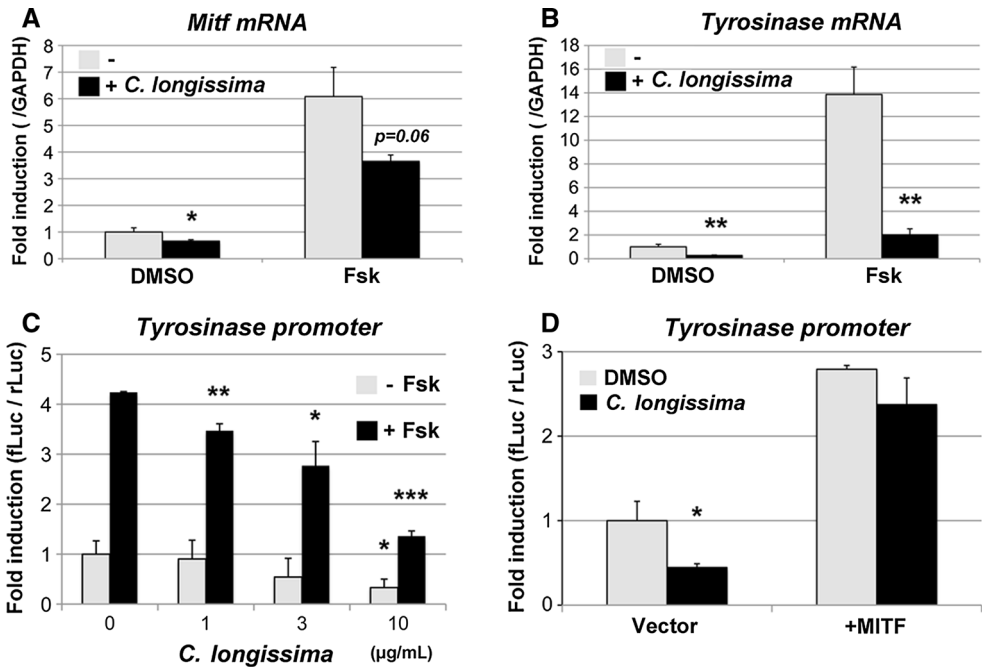


Fig. 3 *Mitf* expression is critical for *C. longissima* extract-dependent suppression of *tyrosinase* promoter activity. **a** Total RNA was extracted from B16F10 melanoma cells that had been treated with Fsk (15 μM for 3 h) together with the *C. longissima* extract (3 μg/mL), and used as templates for quantitative PCR to measure MITF mRNA ($n = 3$, means and SD are indicated), $*p < 0.05$. **b** The same experiment was performed for *Tyr* mRNA in 48-h treated cells, $**p < 0.01$. **c** pGL4-mouse tyrosinase promoter reporter (fLuc) was transformed into B16F10 melanoma cells together with an internal TK reporter (rLuc). After 24 h, Fsk (15 μM) was added to the culture medium and the cells were incubated further for 24 h ($n = 4$). **d** The expression vector for mouse MITF was co-transformed with the pGL4 mouse tyrosinase promoter reporter. After 48 h, reporter activities were measured

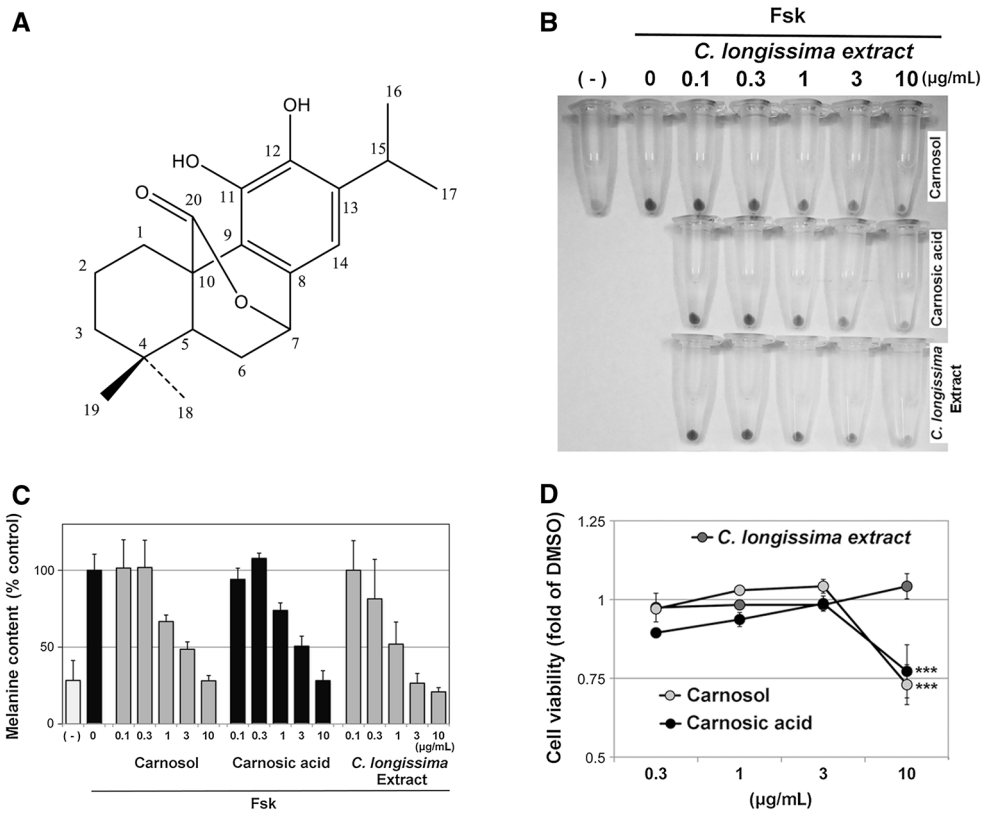


Fig. 4 *C. longissima* extract is abundant in carnosol and carnosic acid. **a** Structure of carnosol. **b** B16F10 melanoma cells were treated with Fsk (15 μM) for 48 h together with indicated concentrations of carnosol, carnosic acid, and the *C. longissima* extract and melanin content was measured (**c**). **d** Mouse B16F10 melanoma cells (plated on a 96-well plate at 1×10^4 cells per plate) were treated with extract, compounds, or DMSO alone for 48 h. Cell viability was estimated by measurement of cellular NADH with the WST-8 kit. Values (OD) were normalized to that of DMSO alone (0 μg/mL compounds). $n = 4$, means and SD are shown. $^{***}p < 0.001$

binding protein p300 (CBP/p300) [21], kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) [19], p38 [22], β-catenin [23], nuclear factor κB-matrix metalloprotainase-9 [24, 25], transient receptor potential ankyrin 1 [26], and AMP-activated kinase (AMPK) [27]. CBP/p300 acts as a CREB coactivator, upregulates MITF gene expression in melanocytes, and promotes MITF-dependent transcription [28]. Carnosol suppressed CBP/p300 on the cyclooxygenase-2 promoter in human 184B5/HER breast epithelial cells [21]. In contrast to CBP/p300, AMPK is proposed to suppress CREB activity in the liver via the phosphorylation-dependent inhibition of other coactivator CRTCs (CREB-related transcription coactivators) [29], and CRTC1 promotes MITF gene expression in melanocytes [6]. Although the AMPK activator metformin was found to suppress CREB activity in human melanocytes by reducing cAMP levels [30], the metformin-dependent suppression of melanogenesis did not require AMPK, suggesting that AMPK is not essential for the suppressed melanogenesis found for the *C. longissima* extract. β-Catenin also acts as a co-activator of the LEF/TCF complex, which up-regulates MITF gene expression in Wnt signaling [31]. Carnosol binds β-catenin and promotes its degradation [23]. These reports suggest that carnosol and carnosic acid suppress MITF gene expression through multiple cascades.

In addition to these well-known cascades regulating *Mitf* promoter activity, overexpression of Nrf2 was found to suppress MITF gene expression [32]. In unstressed cells, Nrf2 binds to Keap1, a recruiter of Nrf2 to the proteasome complex, leading to Nrf2 degradation [33]. Keap1 has free thiol moieties that act as sensors of redox status. Once the thiol moieties are oxidized, Keap1 is degraded and releases Nrf2, which induces Nrf2-dependent transcription of anti-oxidative molecules [34]. Carnosic acid attacks the thiol moieties of Keap1 and activates the Nrf2 pathway [19].

The p38 pathway also has been reported to regulate Nrf2 both positively [35] and negatively [36]. In addition, p38 is a component of a UV-induced stress signal that promotes melanogenic programs. Indeed, carnosol up-regulated p38 phosphorylation in B16F10 melanoma cells [37], suggesting the complex roles of p38 in melanogenesis.

Rosemary is rich in carnosol and carnosic acid, and is used for a variety of purposes [17], including in foods [38]. By evaluating anti-melanogenic activity, we found that the ethanol extract of *C. longissima* leaf was also rich in these substances and strongly inhibited MITF gene expression.

Although *C. longissima* is currently in danger of extinction in Japan [13], we have successfully cultured this plant on the experimental farm operated by the Tanegashima Division of the Japan National Institute of

Biomedical Innovation, Health and Nutrition. This suggests that *C. longissima* may be an attractive source of skin-whitening compounds used in the cosmetic industry. Further study is required to elucidate the molecular mechanism by which the *C. longissima* extract inhibits melanogenesis.

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Compliance with ethical standards

Conflict of interest MY and AK are employees of Momotani-juntenkan Co. Ltd., which paid 300,000 JP Yen: 2012 towards the costs of this study. Other contributors declare that they have no conflict of interest.

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