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# Improvement of Lentigines by Oral Crocetin Administration and Examination of Its Mechanism of Action

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## Abstract

We investigated the effect of crocetin, a carotenoid present in the fruits of *Gardenia jasminoides*, on the improvement of lentigines in the skin and examined its mechanism of action. Subjects consumed an experimental meal consisting of a drink containing 7.5 mg of crocetin daily after dinner for 8 weeks. We examined the effects of oral ingestion of crocetin on the improvement of the size of lentigines on the skin. In an epidermis model in which crocetin was added at concentrations of 1 μg/mL and 2 μg/mL and cultured for 14 days, melanin was significantly suppressed compared with the control. Investigation of the mechanism of action via a cultured human epidermis model and cultured pigment cells revealed that crocetin decreased melanocortin receptor subtype 1 of melanocytes at the messenger ribonucleic acid level and inhibited the stimulatory action of melanocytes via melanocyte-stimulating hormone. The results of the study concluded that crocetin inhibits melanin synthesis by controlling the synthesis of tyrosinase and tyrosinase-related protein-1 via the suppression of melanocortin receptor subtype 1 expression.

**Keywords:** Crocetin; Melanin; Tyrosinase; Tyrosinase-related protein-1; Oral administration; Pigmentation

**Abbreviations:** ANOVA: Analysis of Variance; DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal Bovine Serum; MITF: Microphthalmia Transcription Factor; MSH: Melanocyte-Stimulating Hormone; PBS: Phosphate-Buffered Saline; PCR: Polymerase Chain Reaction; SCF: Stem Cell Factor; SD: Standard Deviation; DMSO: Dimethyl Sulfoxide; IgG: Immunoglobulin G; RNA: Ribonucleic Acid; UV: Ultraviolet

## Introduction

Local lentigines manifest in the face and hands with age and are often accelerated by exposure to Ultraviolet (UV) rays. Melanin leading to pigmentation is catalyzed by tyrosinase within intracellular organelles, melanosomes, of melanocytes, and is sent to the keratinocytes of the skin in conjunction with Tyrosinase-Related Protein-1 (TRP-1) [1-3]. While melanin in the epidermis plays a role in protecting the skin from UV rays, this exposure can lead to unusual pigmentation in the form of lentigines, which are skin lesions that develop over several years. As such, the identification of foods that can prevent the formation of lentigines is desirable. Although UV exposure increases melanin in the skin, melanocytes, which are scattered in the basal epidermal layer, consequently grow in size. It has been observed that dendrites remarkably extend and quickly deliver melanosome to the surrounding keratinocytes [4]. Although melanocytes produce melanin, not only melanocytes but also the influence of the surrounding keratinocytes promotes melanin generation by UV rays [5,6]. Melanocytes constitute the surrounding keratinocytes and the close signal network between the cells. Stressful stimuli, such as UV rays and inflammation, can cause melanocytes to become easily influenced by Stem Cell Factor (SCF), a cytokine, and Melanocyte-Stimulating Hormone (MSH), the hormone that promotes melanin synthesis. Melanocytes are activated by receiving these factors through various signal transduction systems that take place within the melanocytes [7,8]. It has been reported that there are individual differences in susceptibility to the appearance of lentigines on the skin, and Melanocortin Receptor Subtype 1 (MC1R) varies in people with melasma and freckles. It is known that MC1R plays an important role in the generation of lentigines and freckles [9].

The fruits of *Gardenia jasminoides* are used as an herbal medicine known as Gardeniae fructus (sannshishi), are used to treat maladies, such as jaundice, in Japan. Crocetin (Figure 1), a carotenoid carboxylic acid, naturally exists and belongs to the xanthophylls, a type of carotenoid, of non-provitamin A. It is used to color various foods because of the yellow pigment present in the fruits of *Gardenia jasminoides* of the Gardenia genus of the Rubiaceae family. It is also used as a food component that is useful for the maintenance and improvement of health. Research suggests that oral ingestion of crocetin can decrease physical fatigue in healthy individuals [10]. Moreover, effects of suppressing the obstacle of the eye by light [11] and improving sleep disorders [12] have been reported. Furthermore, it is reported that crocetins are easily absorbed by the body and that concentrations in the blood reach a maximum at approximately 4 h after absorption, as opposed to lutein, which belongs to the xanthophylls, or β-carotene, which belongs to the carotenes and is the same type of carotenoid as crocetin [13].

This paper aimed to examine the mechanism of action of crocetin extracted from the fruits of *Gardenia jasminoides* in improving

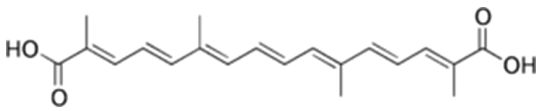


**Figure 1:** Chemical structure of crocetin.

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lentigines in the skin via (1) a study on adult women and (2) examination of cultured cells.

## Materials and Methods

### Participants

The subjects included 44 healthy women (mean age, 41.3 ± 5.5 years; range, 30–50 years) who had two or more lentigines on the cheeks.

### Human study: ingestion of crocetin-containing drink

This study was designed as open-labeled, before-after trials. Subjects consumed a test meal, which consisted of a drink containing 7.5 mg of crocetin, daily after dinner for 8 weeks. Images of conspicuous lentigines in the subjects' cheeks were taken using a digital microscope (Hirox Co., Ltd., Tokyo, Japan) before and after ingestion of the test meal. Image analysis computed and estimated the surface area of the lentigines. This study received approval from the Ethics Committee of Shiba Palace Clinic, and was conducted in accordance with the Declaration of Helsinki. Subjects were fully informed regarding the content and method of this study prior to obtaining their written informed consent.

### Examination of the mechanism of action in a human three-dimensional (3D) epidermis model

The influence of crocetin on melanin generation was investigated using a 3D human epidermis model (J-Tec Industries, Inc., Aichi, Japan). Crocetin of no less than 95% purity (Riken Vitamin Co., Ltd.) was used for this examination of cultured cells. The influence of crocetin on amounts of tyrosinase messenger ribonucleic acid (mRNA), TRP-1, microphthalmia transcription factor (MITF), and MC1R were also investigated. The model was cultured for 14 days in a culture medium (50 ng/mL MSH, Sigma-Aldrich, St. Louis, MO, USA) to which crocetin at concentrations of 0.5 μg/mL, 1 μg/mL, 2 μg/mL in dimethyl sulfoxide (DMSO) or DMSO (control) were added. Culture medium exchange was performed every 2 days. The melanin value of the 3D epidermis model was measured using Mexameter MX 18 (Courage+Khazaka Electronic GmbH, Köln, Germany), and melanocytes were observed under inverted microscope (Olympus Corporation, Tokyo, Japan). Mexameter MX 18 is an apparatus that quantifies melanin at two wavelengths (660 and 880 nm) as an index of melanin. To measure the amounts of tyrosinase, TRP-1, MITF, and MC1R mRNA, the model was cultured for 2 days in the culture medium (50 ng/mL MSH) to which crocetin at concentrations of 1 μg/mL and 2 μg/mL in DMSO or DMSO (control) were added. RNA was then extracted, and the amounts of mRNA were analyzed using real-time polymerase chain reaction (PCR). RNA extraction was performed using an RNA extraction kit (RNeasy Protect Mini Kit, QIAGEN K.K., Tokyo, Japan), and real-time PCR was performed using ABI PRISM 7900HT apparatus (TaKaRa One Step SYBR PrimeScript RT-PCR Kit II, Takara bio Inc., Shiga, Japan). The primers of tyrosinase, TRP-1, MITF, MC1R, and β-actin were purchased from Qiagen.

### Measurement of tyrosinase activity, tyrosinase and MC1R mRNA, protein levels of MC1R, and TRP-1 in cultured pigmented cells

B16 melanoma cells were seeded in a 96-well microplate with 10,000 cells per well and were cultured in a 10% bovine serum containing Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific Inc., Waltham, MA, USA). The following day, B16 melanoma cells were cultured for 3 days with added crocetin (at concentrations of 0.1 μg/mL, 1 μg/mL, and 10 μg/mL in DMSO) or DMSO (control) in a 10% bovine serum containing DMEM. The number of cells and tyrosinase activity were measured using the Hoechst 33342 and L-dopa (L-DOPA) method [14]. The cells were added to 10 μg Hoechst 33342 (10 μg/mL), and fluorescence intensity (excitation at 360 nm, fluorescence 460 nm) was measured after 1-h incubation at 37°C. After removing the culture medium, it was washed once in phosphate-buffered saline (PBS), and 90 μL of 1% Triton-X/PBS was added for cell membrane destruction. The reaction was initiated by the addition of 10 μL, 10 mM L-DOPA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and incubation at 37°C. At the start of the reaction and after 2 h, the absorbance of 475 nm was measured with a microplate leader (Multi-Detection Microplate POWERSAN HT, BioTek Instruments Inc., Winooski, VT, USA). The change in absorbance of 475 nm was assumed to be due to the tyrosinase activity of the cells.

Moreover, B16 melanoma cells were cultured in a 35-mm dish with 60,000 cells per well, and crocetin (at concentrations of 0.1 μg/mL, 1 μg/mL, and 10 μg/mL in DMSO) or DMSO (control) was exposed on the following day and cultured for 2 days. RNA was extracted, and the amounts of tyrosinase, TRP-1, MITF, and MC1R mRNA were analyzed using real-time PCR. The primers of tyrosinase, TRP-1, MITF, MC1R, and glyceraldehyde 3-phosphate dehydrogenase were purchased from Qiagen. Moreover, B16 melanoma cells were seeded in a chamber slide (AGC Techno Glass Co., Ltd., Shizuoka, Japan), with 60,000 cells per well and cultured using 10% Fetal Bovine Serum (FBS) containing DMEM. The following day, it was cultured for 2 days in 10% FBS containing DMEM, to which crocetin (at concentrations of 0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL in DMSO) or DMSO (control) was added. After being fixed by a 4% paraformaldehyde phosphoric acid buffer solution and being washed twice with PBS, it was stained with anti-MC1R antibody (Abcam, Cambridge, UK), anti-TRP-1 antibody (TMH-2) [15], and a secondary antibody [Goat anti-rat immunoglobulin G (IgG) conjugated Alexa488, Goat anti-rabbit IgG conjugated Alexa568, Thermo Fisher Scientific Inc., Waltham, MA, USA]. Finally, it was observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

### Statistical analysis

In the human study, after calculating the average surface area and standard deviation (SD) of lentigines, paired t-tests were performed. In the cultured cell experiment, after calculating the average value and SD, ANOVA and unpaired t-tests were performed to compare the experimental and control groups. Statistical significance was set at $P<0.05$ or $P<0.01$.

## Results

### Effect of crocetin-containing drink on the surface area of lentigines

The conspicuous areas of lentigines in the subjects' cheeks were calculated via image analyses pre- and post-ingestion of a crocetin-containing drink. Results indicated that the area of facial lentigines significantly decreased after the subjects consumed crocetin daily for 8 weeks (Table 1). A representative example of lentigines pre- and post-crocetin ingestion is shown in Figure 2. The size and the density of lentigines significantly decreased after crocetin ingestion for 8 weeks.

### Examination of the mechanism of action in the 3D human epidermis model

The shape of microscopic images and melanin index values

computed by Mexameter measured in the 3D human epidermis model and cultured for 14 days with or without crocetin (at concentrations of 0.5 μg/mL, 1 μg/mL, and 2 μg/mL) are shown in Figure 3. In the epidermis model, in which crocetin was added at concentrations of 1 μg/mL and 2 μg/mL and cultured for 14 days, melanin was significantly suppressed compared with the control.

In addition, 50 ng/mL MSH-containing medium was cultured for 2 days after examining the effect of crocetin on TRP-1, MITF, MC1R, and tyrosinase mRNA expression (at concentration of 1 μg/mL and 2 μg/mL). The amount of MC1R mRNA significantly decreased compared to the control at crocetin concentrations of 1 μg/mL and 2 μg/mL crocetin. The amount of both tyrosinase and TRP-1 mRNA significantly decreased compared to the control in the presence of 2 μg/mL crocetin (Figure 4). On the other hand, levels of MITF mRNA did not decrease under crocetin concentrations of 1 μg/mL and 2 μg/mL (Figure 4).

### Effect of crocetin on tyrosinase activity, tyrosinase and MC1R mRNA, protein levels of MC1R, and TRP-1 in cultured pigmented cells

As a result of culturing B16 melanoma cells for 3 days at crocetin concentrations of 0.1 μg/mL, 1 μg/mL, and 10 μg/mL, tyrosinase activity was significantly inhibited without affecting the number of cells (Figure 5A). Moreover, the amount of both tyrosinase and MC1R mRNA significantly decreased at concentrations of 1 μg/mL and 10 μg/mL for 2 days (Figure 5B). Furthermore, as a result of investigating MC1R and TRP-1 by indirect fluorescent antibody staining methods, immunoreactive MC1R and immunoreactive TRP-1 decreased at crocetin concentration of 1 μg/mL (Figure 5C).

## Discussion

The fact that the surface area of lentigines decreased following ingestion of the test drink suggested that lentigines improved by crocetin ingestion. Because tyrosinase and TRP-1 are known to increase as a result of the stimulation of MSH in melanocytes, the mechanism of action by which crocetin improved lentigines is thought to be a decrease in MC1R of melanocytes at the mRNA level, which in turn decreases MSH bound to its receptor in melanocytes. Various intracellular signaling pathways participate in the process, leading to expression of the tyrosinase gene, which underwent phosphorylation of MITF due to the acceptance of the melanocytes activated factor. In the case of MSH, which is one of the common melanocyte activated factors, signal transduction occurs in melanocytes through MC1R, which is a G-protein-coupled receptor that exists in cell membranes. The gene and protein expressions and enzyme activity of tyrosinase thus increases,

| | Unit | n | Pre-intake | Post-8-week-intake | Statistical significance |
|---|---|---|---|---|---|
| | | | Mean ± SD | Mean ± SD | *P*-value |
| Surface area of lentigines | $mm^2$ | 44 | 0.830 ± 0.637 | 0.689 ± 0.596 | <0.05 |

*A drink including 7.5 mg crocetin was consumed daily after dinner for 8 weeks. Paired t-test was used to test the statistical significance. SD: Standard Deviation

**Table 1:** The surface area of lentigines pre- and post-crocetin ingestion* in healthy female participants.

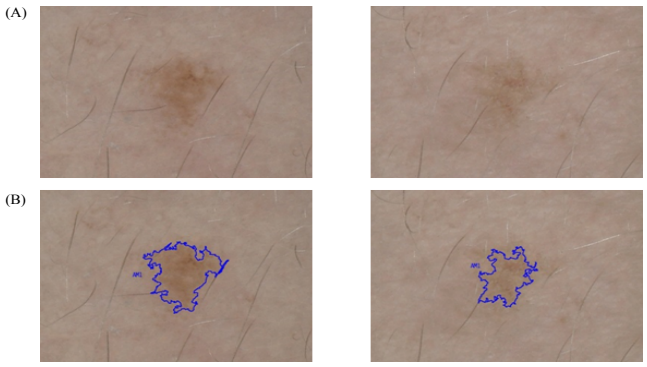


**Figure 2:** An example representation of lentigines at pre- and post-8-weeks crocetin ingestion. (A) Microscope image of a 40-year-old woman's face; left: pre-ingestion; right: post-ingestion. (B) The outline of lentigines is depicted with a blue line; left: pre-ingestion; right: post-ingestion. Results indicated that the size and depth of lentigines decreased post-crocetin ingestion.

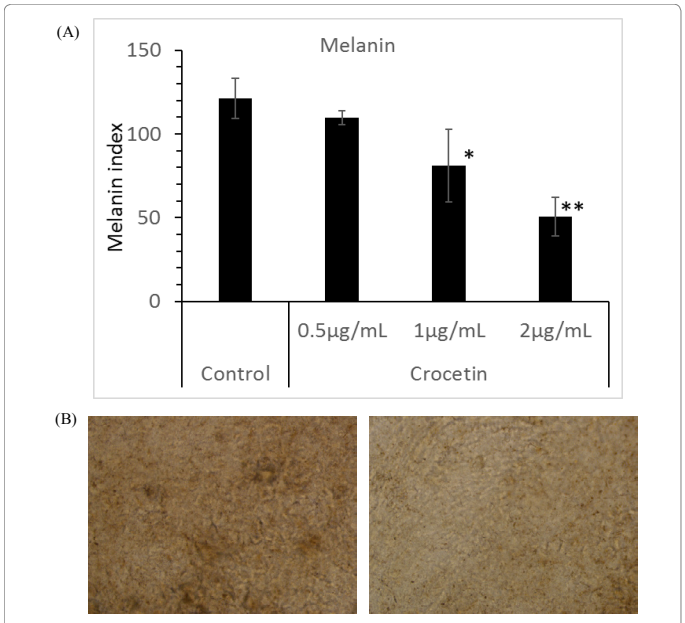


**Figure 3**: (A) Relative melanin values of 3D human epidermis model cultured for 14 days with or without crocetin; n=3, mean ± SD. *$P$<0.05 vs. control; **$P$<0.01 vs. control. (B) The representation features of microscopic image; left: control; right: 2 μg/mL crocetin.

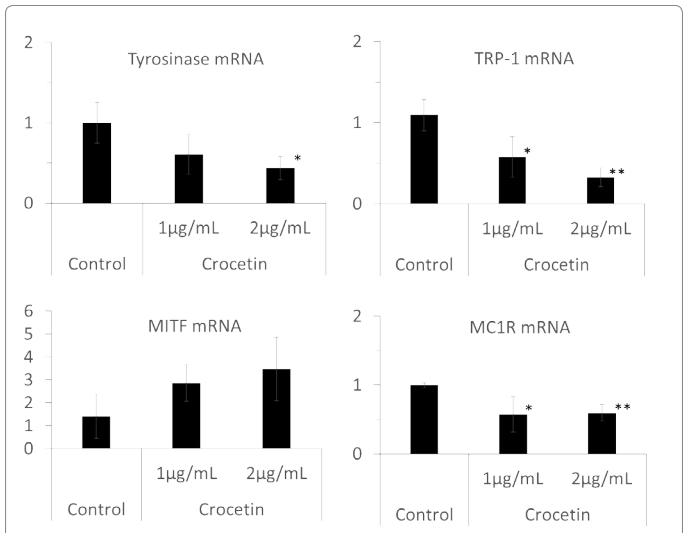


**Figure 4:** Amount of mRNA of tyrosinase, TRP-1, MITF, and MC1R in 3D human epidermis model cultured for 2 days with or without crocetin; n=3, mean ± SD. *$P$<0.05 vs. control, **$P$<0.01 vs. control.

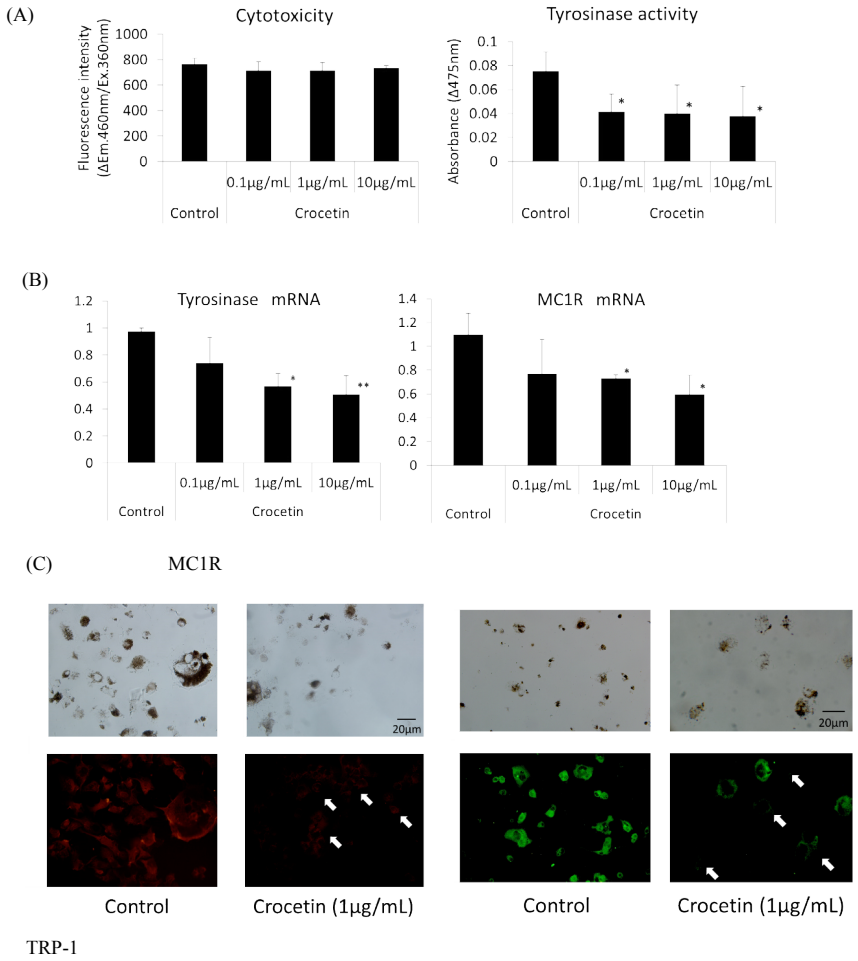


**Figure 5:** The number of cells and tyrosinase activity of melanocytes cultured with or without crocetin; n=4, mean ± SD. (A) $^{*}P<0.05$ vs. control. (B) Amount of mRNA of tyrosinase, TRP-1, MITF, and MC1R in 3D human epidermis model cultured for two days with or without crocetin; n=3, mean ± SD. $^{*}P<0.05$ vs. control, $^{**}P<0.01$ vs. control. (C) Bright field image of cells, fluorescent image of immunoreactive MC1R and immunoreactive TRP-1, and white arrow show decreasing immunoreactivity.

and melanin generation is induced [16]. Melanocytes stimulate by MSH activate adenylate cyclase, leading to increased intracellular cyclic adenosine monophosphate concentration, which activates a specific pathway and promotes phosphorylation of MITF [17]. MITF strongly affects melanocyte survival, and it has been reported that melanocytes can no longer survive after inhibition of MITF mRNA expression [18]. Because crocetin is unable to decrease the quantity of MITF mRNA, it is not believed that crocetin decreases lentigines due to toxicity of melanocytes. In the current study, crocetin did not affect the amount of mRNA in either SCF or pro-opiomelanocortin, which is a precursor of MSH (data not shown). Crocetin directly acts on melanocytes and is considered to suppress melanin synthesis in a human epidermis model. The plasma concentration of crocetin when taking 7.5 mg of crocetin orally is reported to be approximately 0.1 μg/mL [13]. This experiment found that tyrosinase activity is significantly inhibited with an intake of 0.1 μg/mL crocetin. Therefore, it is believed that this experiment is valid in terms of the mechanism of action of crocetin.

Improved brightness of the skin after oral ingestion of the xanthophylls lutein and zeaxanthin has been previously reported [19]. Fucoxanthin and β-cryptoxanthin, which are also xanthophylls, not only reduce the amount of MC1R mRNA but also decrease mRNA of prostaglandin E receptor 1 [20,21]. In addition, it has been reported that crocetin reduces oxidative stress via antioxidant activity [22] and inhibits inflammation induced by lipopolysaccharides [23]. Further, if weak inflammation persists in the skin, stimulation may result in deeper or larger lentigines. The decreased size of lentigines induced after ingestion of crocetin in the current study also may have been due to crocetins anti-inflammatory effects. Moreover, crocetin absorption may occur at wavelengths within the range of UVB and UVA and may protect the skin from damage by UV rays. On the other hand, retinoic (vitamin A) acid is reported to cause thinning of lentigines [24]. However, it is not believed that crocetin showed its effect via vitamin A-like action on lentigines because crocetin is not converted into vitamin A in the body. In addition, previous research reported no side effects of vitamin A overdose, without lutein being converted to vitamin A [25]. It is, therefore, believed that there are no side effects accompanied by vitamin A overdose because water solubility of crocetin is higher than that of lutein, it does not accumulate in the tissue, and is not converted to vitamin A.

Based on the above findings, we conclude that oral ingestion of crocetin, a carotenoid contained in the fruits of *Gardenia jasminoides,* can be expected to improve lentigines on the skin. Findings indicated

that crocetin ingestion led to decreased MC1R of melanocytes at the mRNA level and inhibited the action of melanocytes of MSH, which is thought to be due to the inhibition of melanin synthesis by crocetin. This in turn is expected to suppress the synthesis of both tyrosinase and TRP-1, representing one of crocetin's mechanisms of action on lentigines.

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