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RESEARCH ARTICLE

ROS-scavenging and Anti-tyrosinase Properties of Crocetin on B16F10 Murine Melanoma Cells

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Abstract: ***Background*:** *Crocus sativus* (Iridaceae) has been traditionally used in the Iranian folk medicine and as a culinary additive. Major components of the plant that are responsible for biological properties are saffranal, crocin, picrocrocin and crocetin. Although the level of crocetin is not high, some of the important activities of saffron such as antioxidant activity have been attributed to crocetin.

***Objective*:** In the present study, we investigated the effects of crocetin on melanogenesis in B16 melanoma cells.

***Methods*:** The effect of crocetin on intracellular and mushroom tyrosinase activity and the content of melanin was evaluated spectrophotometrically. Tyrosinase and Microphthalmia-Associated Transcription Factor (MITF) protein levels were compared between Crocetin-treated and control cells after western blot analysis. The anti-oxidative activity of crocetin was also investigated.

***Results*:** Crocetin could inhibit mushroom tyrosinase activity and lower the amount of melanin in B16 melanoma cells. Protein levels of tyrosinase and MITF were also decreased by crocetin. Crocetin also showed antioxidant activity and depleted cellular Reactive Oxygen Species (ROS) content but had no cytotoxicity in alamarBlue® assay.

***Conclusion*:** Taken together, decreased tyrosinase activity, melanin content, tyrosinase and MITF proteins levels, and ROS production showed the inhibition of melanogenesis in B16F10 cells by crocetin. Hence, crocetin could be suggested as a potential dermatological whitening agent in skin care products.

Keywords: *Crocus sativus*, crocetin, Iridaceae, anti- tyrosinase, melanogenesis, B16F10 murine melanoma cells.

1. INTRODUCTION

Melanin is the skin pigment of mammals which is synthesized by melanocytes. In humans, melanin determines the color of skin, hair and eyes. Melanin is synthetized in melanosomes - melanocyte cell membrane binding granules - and is transferred to keratinocytes through a physiological process called melanogenesis [1]. The key enzyme in melanin biosynthesis found inside melanosomes is a copper-containing enzyme, tyrosinase, which catalyzes two separate reactions [2]. In the process of melanin formation, hydroxylation of a monophenol is followed by the conversion of an o-diphenol to o-quinone by tyrosinase activity. The gene encoding tyrosinase enzyme is *TYR* [3].

Oxygen free radicals are a subset of Reactive Oxygen Species (ROS) and are generated from exogenous and endogenous origins during normal metabolism and biochemical processes of the tissues of living organisms. As long as the amount of free radicals does not exceed the antioxidant capacity, they have positive effects on health and human development. Considering the role of ROS in various human diseases, antioxidants are essential to combat damaging effects of the natural physiological process of oxidation in the tissues [4]. UV-induced production of ROS is one of the factors which can increase the biosynthesis of melanin, melanocyte proliferation and DNA damage. Thus, natural sources of antioxidants with anti-tyrosinase activity contribute to the mitigation of skin damages due to melanogenesis [2]. Phytochemicals can serve as precursors for the synthesis of compounds with lower toxicity compared with synthetic compounds [5, 6]. Crocetin is a natural carotenoid and one of the components of saffron (*Crocus sativus* L.). Crocetin is a symmetric di-carboxylic acid diterpene with 7 double bonds and 4-methyl groups. About 94% of the total amount of crocetin in saffron is present in the glycosylated form (crocin) and 6% of crocetin is in the free form [7]. Considering the proven antioxidant activity of saffron in many studies and the important role of crocetin as an antioxidant [8], this study was conducted to study the anti-tyrosinase effects of crocetin. This project was designed to study the effects of various concentrations of crocetin in inhibiting tyrosinase activity and to assess the antioxidant and ROS-scavenging effects of this phytochemical in B16F10 melanoma cells. We also evaluated the possible effect of crocetin in modulating hyperpigmentation and inhibiting the pathway of melanogenesis.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

AlamarBlue® (resazurin) from Sigma (Saint Louis, MO, USA); RPMI-1640 and FCS from GE Healthcare Life Sciences; β -actin

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and anti-rabbit IgG and HRP linked antibody from Cell Signaling Technology (Boston, USA); Tyrosinase (H-109) and MITF (H-50) rabbit polyclonal antibody from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA) and Western blotting detection reagent from Bio-RaD (USA); α -melanocyte stimulating hormone, 3,4-dihydroxy-L-phenylalanine, mushroom tyrosinase, protease inhibitor cocktail, phosphatase inhibitor cocktail, phenylmethylsulfonyl fluoride and QuantiPro BCA Assay Kit from Sigma (Steinheim, Germany); all solvents were analytical grade and were purchased from Dr. Mojallali Lab. (Tehran, Iran).

2.2. Preparation of Crocetin

Crocetin was extracted from crocin as previously described [9]. First, dried stigmas of saffron were washed with ice-cold ethanol 80% in eight steps. The supernatant was kept at -5ºC for 24 days in a dark place. Crocin crystals (yield: 10-15%) were then separated and washed with acetone. Crocin was then hydrolyzed in HCl 33%. After 2 min contact with N2 the solution was kept at 50ºC for 1 h and then centrifuged with 17 ml distilled water (3000 rpm for 10 min). The supernatant was then separated (yield 10-12%). The structure of crocetin is presented in Fig. (**1**).

2.3. Cell Culture

B16F10 melanoma cells were obtained from Pasteur Institute (Tehran, Iran) and maintained in RPMI-1640 medium with 10% v/v Fetal Bovine Serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The stock solution of crocetin (50 mM) was prepared in DMSO and kept at -40ºC. The final concentration of DMSO was set at 0.05% which was non-toxic with no significant effect on melanogenesis. Kojic acid (2 mM) was used as positive control in all experiments. Cells were cultured in multi-well plates at a density of 10^5 cells/ml.

2.4. Melanin Quantification

B16F10 melanoma cells were seeded at a density of 2×10^5 cells per well in 12-well culture plates and incubated for 24 h. The cells were then incubated with and without crocetin (0-32 µM) for 24 h. Melanin content was measured as described previously [10]. After treatment, the cells were collected using trypsin and washed twice with PBS. The melanin content of cells was then extracted using 2 M NaOH at 100°C for 30 min. The melanin content was measured at 405 nm using a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA).

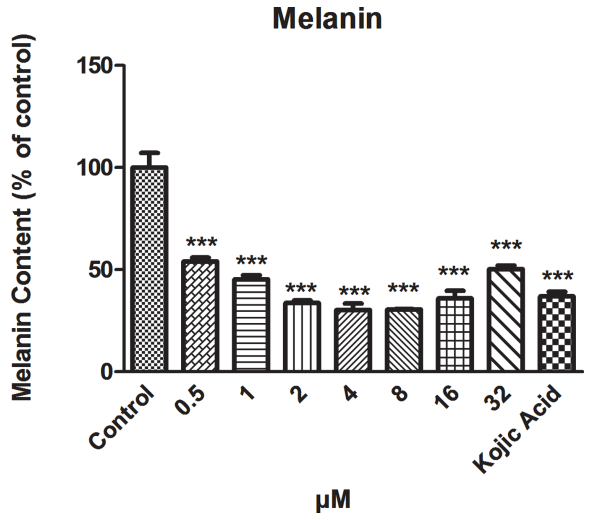

Fig. (1). Structure of crocetin and the pathways influenced by crocetin in melanogenesis process.

2.5. Cell Viability Assay

The alamarBlue® assay t indicates the reducing power of the living cells in converting resazurin to resorufin. This reduction occurs in the cytosol of the living cell. Resazurin is a non-toxic and cell permeable compound that is blue in color and is virtually non-fluorescent but resorufin is a compound that is red in color and highly fluorescent. Resazurin is continuously convert to resorufin in the living cells and the rate of increase in the total fluorescence intensity is correlated with the number of living cells [11]

About 10^4 B16F10 melanoma cells were seeded in each well of 96-microwell plate and treated with various concentration of crocetin (0-32 µM). After 4 h of incubation, the absorbance of alamarBlue® was measured at 570 nm and 600 nm. Each experiment was done in triplicate.

2.6. Mushroom Tyrosinase Activity Assay

The activity of mushroom tyrosinase in oxidation of L-DOPA was measured by spectrophotometry as described previously [12] with some modifications. Briefly, 160 µL of 5 mM L-DOPA (in 100 mM sodium phosphate buffer, pH 6.8) and 20 µL of the same buffer with and without crocetin (0-32 µM) were mixed with 20 µL of mushroom tyrosinase (200 units/mL) and then incubated at 37°C for 30 min. The absorbance at 475 nm was measured using a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA).

2.7. Cellular Tyrosinase Activity Assay

The oxidation of DOPA to DOPAchrome as an indicator of tyrosinase activity was analyzed by spectrophotometry. B16F10 melanoma cells (2×10^5) were plated in each well of 12-well plate and incubated for an overnight. After treating of cell with or without crocetin (0-32 µM) for 24 h, the cells were detached using trypsin and were then washed with PBS twice and lysed with 100 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 10,000 rpm for 20 min at 4°C. 100 µL of each lysate (each containing 100 mg of the protein) was mixed with 100 µL of 5 mM DOPA in 96-well plate and incubated at 37°C for 2 h, followed by the measurement of absorbance at 475 nm with a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA).

2.8. Cellular ROS Level Determination

About 2×10^4 B16F10 melanoma cells were cultured in 96-well plates and treated with or without crocetin (0-32 µM) for 24 h. Following cell exposure to 24 mM H_2O_2 at 37°C for 30 min, 2', 7'-dichlorofluorescein diacetate (DCFH-DA) was added to each of the wells and incubated for the next 30 min. The amount of ROS was measured according to fluorescence intensities of DCF at the excitation wavelength 504 nm and emission wavelength of 524 nm using a Synergy H4 Hybrid Multi-Mode fluorescent Microplate Reader (BioTek, Winooski, USA) [13].

2.9. Western Blotting Analysis

B16F10 melanoma cells were cultured in 25 cm^2 flasks with and without crocetin (0-32 µM) for 24 h. The cells were then lysed in a buffer (50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-Glycerophosphate, 10 mM β-mercaptoethanol, 1 mM sodium orthovanadate and 0.1% deoxycholic acid sodium salt). Equal amount of proteins (50 µg) were loaded on 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 2 h in 10% skim milk in TBST (20 mM Tris-HCl pH 7.4, 100 mM NaCl and 0.1% Tween 20) buffer at room temperature. After washing with TBST buffer, the membrane was then

incubated overnight with a primary antibody: rabbit anti-tyrosinase antibody (1:300) or anti-MITF antibody (1:300) (Santa Cruz Biotechnology, CA). After rinsing with TBST buffer for 5 times, the membranes were incubated for 2 h with anti-rabbit IgG (1:2000) as the secondary antibody (Cell Signalling). Rinsing 5 times with TBST buffer was repeated and the protein bands were detected using the ECL or ECL Prime Western Blotting Detection System (BioRaD, USA). Anti-β-actin antibody was used as the loading control.

2.10. Statistical Analysis

One-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison tests were performed to check for differences between the groups using Prism 5 Software (GraphPad, La Jolla, CA, USA). Data were presented as mean ± SD of three independent experiments (with three replicates for each tested concentration of crocetin). A p-value of < 0.05 was considered as statistically significant.

3. RESULTS

3.1. Effect of Crocetin on Melanin Production and Cell Proliferation

Murine B16F10 melanoma cells were used as the cell model in examining the inhibitory effect of crocetin on melanogenesis. The melanin content of Crocetin-treated B16F10 melanoma cells was considered as the indicator of the effect of crocetin on melanin production. As shown in Fig. (**2**), melanin level was reduced by crocetin. In the next step, the effect of crocetin on B16F10 melanoma cell proliferation was assessed using alamarBlue® assay. As shown in Fig. (**3**), the compound had no significant cytotoxic effect on B16F10 cells at the concentrations used in this study.

3.2. Effect of Crocetin on Mushroom Tyrosinase Activity

To assess the effect of crocetin on tyrosinase activity directly, mushroom tyrosinase assay was performed using L-DOPA as a substrate and mushroom tyrosinase as the enzyme source. Kojic acid, as a positive control, inhibited tyrosinase activity significantly at the concentration of 2 mM. As shown in Fig. (**4**), crocetin exerted a significant inhibitory effect on L-DOPA oxidation by mushroom tyrosinase.

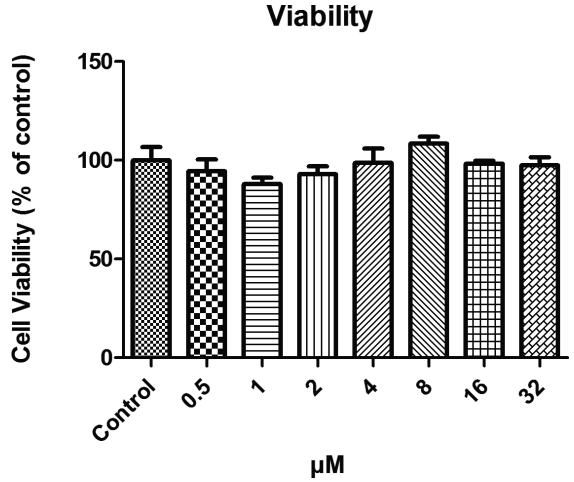

Fig. (2). Effect of crocetin on melanin production in B16F10 Melanoma Cells. B16F10 melanoma cells were incubated without (control) and with 0 to 32 µM of crocetin for 24 h. Kojic acid 2 mM was used as positive control. Results were expressed as percentages relative to control, and are presented as mean±SD for three separate experiments. ***$P < 0.001$ compared to control.

3.3. Inhibitory Effect of Crocetin on B16F10 Melanoma Cellular Tyrosinase Activity

The effect of crocetin on cellular tyrosinase activity was assessed to examine if crocetin affects melanogenesis. As shown in Fig. (**5**), suppression of tyrosinase activity in the cultured B16F10 melanoma cells was not occurred after treatment of cells with crocetin.

3.4. Effect of Crocetin on Cellular ROS Level

The antioxidant capacity of crocetin in cells was assessed by measuring intracellular ROS levels. Cells pretreated with crocetin were exposed to H_2O_2 (24 mM). As shown in Fig. (**6**), crocetin could effectively suppress oxidative stress induced by H_2O_2 in cells.

3.5. Western Blotting of the Expression of Tyrosinase and Related Proteins in Crocetin-treated Cells

The intracellular effect of crocetin on the expression of melanogenic proteins such as tyrosinase and MITF was evaluated using western blot.

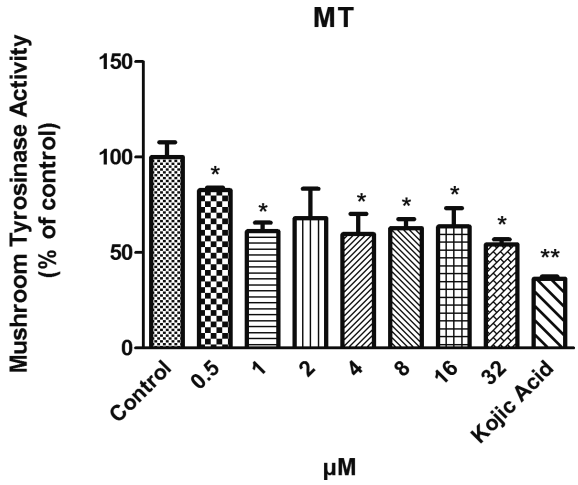

Fig. (3). Effect of crocetin on viability of B16F10 cells. After incubation of B16F10 melanoma cells with various concentrations of crocetin in a 96-well plate for 24 h, cell viability was determined by AlamarBlue® assay. Percentage values in the treated cells were compared with respect to that in the control cells. Data are expressed as mean±SD for three independent experiments.

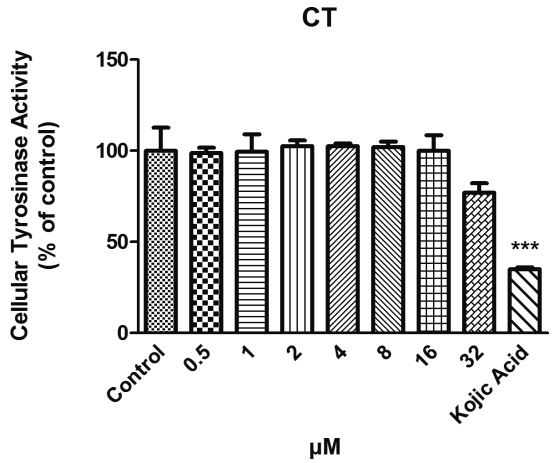

Fig. (4). Effect of crocetin on mushroom tyrosinase activity. Various concentrations of the crocetin and 2 mM of Kojic Acid were incubated with mushroom tyrosinase and L-DOPA at 37°C. Mushroom tyrosinase activity was measured by the change in absorption at 475 nm. Results are expressed as percentages of control. Data are presented as mean±SD for independent triplicate experiments. *$P < 0.05$ and **$P < 0.01$ compared to control.

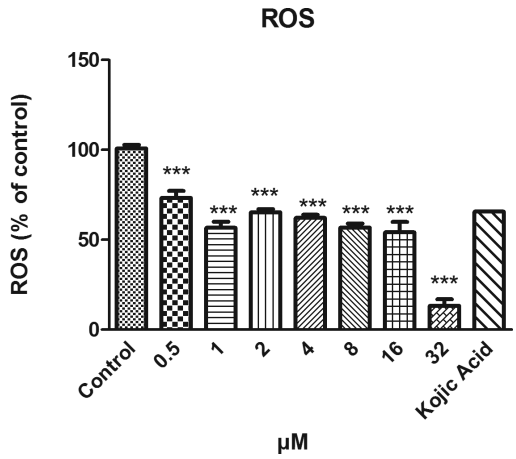

Fig. (5). Inhibitory effect of crocetin on B16F10 melanoma cellular tyrosinase activity. After incubation of B16F10 melanoma cells with various concentrations of the crocetin for 24 h, cellular tyrosinase activity was assessed. Kojic acid 2 mM was used as positive control. Results were expressed as percentages relative to control, and are presented as mean±SD for three separate experiments. ***$P < 0.001$ compared to control.

As shown in Fig. (**7**), tyrosinase and MITF protein levels were significantly decreased by Crocetin, suggesting a potential role of crocetin in down-regulating tyrosinase protein expression and inhibition of melanin production. β-Actin was used as internal control.

DISCUSSION

Considering the safety of natural products, many of the plant-based therapeutic or cosmetic products have emerged with low toxicities. Hydroquinone is one the example that has been almost totally replaced with phytochemicals in skin-whitening products because of melanocyte cytotoxicity and depigmentation of the skin [14]. While plant extracts like licorice are extensively used in skin hyperpigmentation products, there is a need for findings novel whitening compounds [15].

Saffron is a multifunctional plant that has been used for its anti-tyrosinase and antioxidant activity. It is not clear which of the chemicals in the plant is mainly responsible for the anti-tyrosinase activity. Crocetin is the phytochemical found in saffron and possesses antioxidant activity [16, 17]. In the present study, mushroom tyrosinase activity, melanin content in cells and the amount of ROS was significantly decreased following treatment of cells with crocetin while no significant cytotoxicity was observed. Although

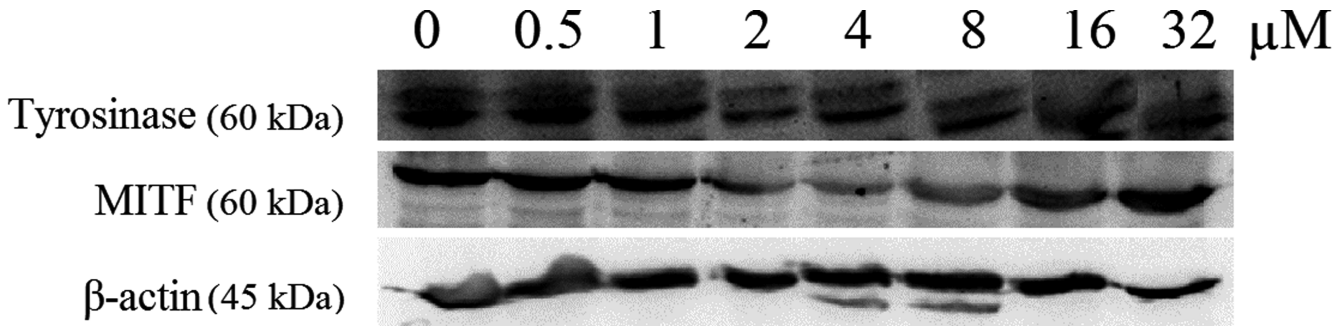

Fig. (6). Effect of crocetin on Cellular ROS level. Antioxidant activities of crocetin by determination of ROS content in B16F10 cells. Cells were treated with various concentrations of the crocetin (0-32 μM) or Kojic acid (2 mM) for 24 h and then the ROS content was measured by the DCF-DA assay. Results were expressed as percentages relative to control, and are presented as mean±SD for three separate experiments. ***$P < 0.001$ compared to control.

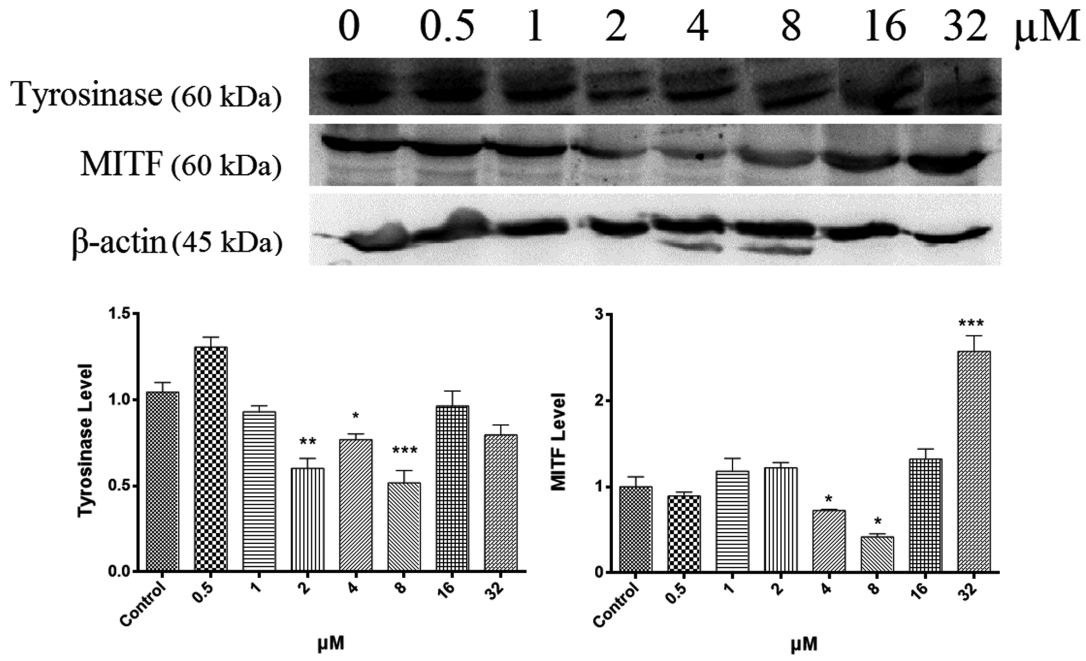

Fig. (7). Western blotting of the expression of tyrosinase and related proteins in crocetin-treated cells. B16F10 melanoma cells were treated with the indicated concentrations of the crocetin for 24 h. Cell lysates were subjected to Western blotting using antibodies against tyrosinase and MITF. The loading control was assessed using β-actin antibody. Data represent for three independent experiments.

the melanin content is slightly increased at 16 and 32 μM but the statistical analysis showed that the difference between "4 μM" versus "16 and 32 μM" were not significant. Moreover, the melanin contents at 16 and 32 μM concentrations were still significantly lower than control, suggesting that Crocetin reduced melanin content even at these concentrations. Also, this was an experimental (preclinical study) and further clinical investigation is required to optimize the concentration which may be applied as whitening agent. Additionally, there are many known natural compounds with dual pharmacological action [18].

To clarify the mechanism of action of Crocetin, we measured protein levels of tyrosinase and MITF. Conversion of tyrosine to dopaquinone is the first step in melanin production that is controlled by Tyrosinase-Related Proteins (TRPs) in the process of melanogenesis. TPRs also increase the conversion and rearrangement of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) which leads to the formation and accumulation of melanin pigments in the skin. TPRs are regulated by MITF in the process of melanin formation in mammalian cells. The results of the present study showed that Crocetin, at the concentrations of 1, 2, 4, 8 and 16 μM, was effective in down-regulating tyrosianse and MITF proteins compared with control cells. Tyrosinase is a key enzyme in the melanogenesis of skin and regulates melanin production. It is supposed that the decrease in melanin synthesis in cultured B16 melanoma cells is related to the inhibition of tyrosinase activity. Alternatively, MITF degradation is a sign of inhibition of TRPs and tyrosinase synthesis. The amount of tyrosinase protein determines the extent of melanin production in cells [19].

Involvement of dioxigen at the dinuclear copper ions at the active site of tyrosinase has been proposed to affect the generation of ROS in the process of melanin formation. Consequently, compounds that can inhibit both free radical formations and tyrosinase activity can enhance skin protection against oxidative stress and hyperpigmentation [20]. Decrease in the protein levels of tyrosinase and MITF by crocetin along with the antioxidant capacities of this phytochemical suggest its use as an anti-melanogenesis agent. The present results showed that the effect of crocetin on melanin content and MITF protein level in B16F10 cells has a biphasic effect. At higher concentration of crocetin (16 and 32 μM), inhibitory effect on the protein levels of pigmentation enzymes and MITF was mitigated while the melanin content, mushroom tyrosinase activity and ROS levels were all decreased. The biphasic effect on the melanogenesis inhibition has also been reported for adenosine [21].

Reported data on the antioxidant and anti-tyrosinase activity of saffron and its components suggest that kaempferol and kaempferol 7-O-beta-d-glucopyranoside are potent radical scavengers while crocusatin-K, crocusatin-L, crocusatin H, crocin-1, and crocin-3 and 4-hydroxy-3,5,5-trimethylcyclohex-2-enone have inhibitory activity against mushroom tyrosinase [22, 23].

Crocetin is a carotenoid found in *Crocus sativus* L. (Iridaceae) which is responsible for the color profile of saffron [24]. Among multiple actions of saffron, antioxidant activity is a proposed mechanism for many of the pharmacological properties like antigenotoxic [25], anti-ischemic [26], antidote [27], anti-Alzheimer, antipruritic, antinociceptive and anti-inflammatory effects [28]. The majority of the pharmacological effects of saffron are due to the presence of crocin (mono and diglycosyl esters of crocetin) [29]. Due to hydrophobic nature of Crocetin, it has higher oral bioavailability and seems to pass more rapidly from cell lipid membrane in comparison with crocin [30]. Hence, crocetin is regarded as the most active free radical scavenger of saffron.

Taken together, decrease in the activity of mushroom tyrosinase as well as reduction of the amounts of melanin, tyrosinase and MITF proteins, and reduction of ROS production suggest the inhibition of melanogenesis in B16F10 cells by crocetin.

CONCLUSION

Hence, crocetin could account for the antioxidant and anti-melanogenesis effects of saffron. Owing to the lack of toxicity of crocetin and antioxidant and anti-tyrosinase activities of this phytochemical on melanoma cells, and the key role of tyrosinase in the biosynthesis of melanin and hyperpigmentation, the use of crocetin as an anti-pigmentation agent is suggested to be tested in future clinical studies.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Sheth, V.M.; Pandya, A.G. Melasma: A comprehensive update: part II. *J. Am. Acad. Dermatol.,* **2011**, *65*, 699-714.

[2] Agar, N.; Young, A.R. "Melanogenesis: a photoprotective response to DNA damage?" *Mutation. Res.,* **2005**, *571*, 121-132.

[3] Gilchrest, B.A. Molecular aspects of tanning. *J. Invest. Dermatol.,* **2011**, *17*(E1), E14-17.

[4] Shafaei-Bajestani, N.; Emami, S.A.; Asili, J.; Tayarani-Najaran, Z. Anti-apoptotic effect of taxodione on serum/glucose deprivation-induced PC12 cells death. *Cell. Mol. Neurobiol.,* **2014**, *34*(8), 1103-1109.

[5] Svobodová, A.; Vostálová, J. Solar radiation induced skin damage: review of protective and preventive options. *Int. J. Radiat. Biol.*, **2010**, *86*, 999-1030.

[6] Kim, Y.J.; Uyama, H. Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cell. Mol. Life. Sci.*, **2005**, *62*(15), 1707-1723.

[7] Bathaie, S.; Farajzade, A.; Hoshyar, R. A review of the chemistry and uses of crocins and crocetin, the carotenoid natural dyes in saffron, with particular emphasis on applications as colorants including their use as biological stains. *Biotech. Histochem.*, **2014**, *89*, 401-411.

[8] Giaccio, M. Crocetin from saffron: an active component of an ancient spice. *Crit. Rev. Food. Sci. Nutr.*, **2004**, *44*(3), 155-172. Review.

[9] Tashakori-Sabzevar, F.; Hosseinzadeh, H.; Motamedshariaty, V.S.; Movassaghi, A.R.; Mohajeri, S.A. Crocetin attenuates spatial learning dysfunction and hippocampal injury in a model of vascular dementia. *Curr. Neurovasc. Res.*, **2013**, *10*(4), 325-334.

[10] Tsuboi, T.; Kondoh, H.; Hiratsuka, J.; Mishima, Y. Enhanced melanogenesis induced by tyrosinase gene-transfer increases boron-uptake and killing effect of boron neutron capture therapy for amelanotic melanoma. *Pigment. Cell. Res.*, **1998**, *11*(5), 275-282.

[11] O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. Investigation of the Alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.*, **2000**, *267*, 5421-5426.

[12] Kim, Y.M.; Yun, J.; Lee, C.K.; Lee, H.; Min, K.R.; Kim, Y. Oxyresveratrol and hydroxystilbene compounds. Inhibitory effect

on tyrosinase and mechanism of action. *J. Biol. Chem.*, **2002**, *277*(18), 16340-16344.

[13] Huang, H.C.; Lien, H.M.; Ke, H.J.; Chang, L.L.; Chen, C.C.; Chang, T.M. Antioxidative characteristics of anisomeles indica extract and inhibitory effect of ovatodiolide on melanogenesis. *Int. J. Mol. Sci.*, **2012**, *13*, 6220-6235.

[14] Parvez, S.; Kang, M.; Chung, H.S.; Cho, C.; Hong, M.C.; Shin, MK.; Bae, H. Survey and mechanism of skin depigmenting and lightening agents. *Phytother. Res.*, **2006**, *20*, 921-934.

[15] Sarkar, R.; Arora, P.; Garg, KV. Cosmeceuticals for hyperpigmentation: What is available? *J. Cutan. Aesthet. Surg.*, **2013**, *6*, 4-11.

[16] Gutheil, W.G.; Reed, G.; Ray, A.; Anant, S.; Dhar, A. Crocetin: an agent derived from saffron for prevention and therapy for cancer. *Curr. Pharm. Biotechnol.*, **2012**, *13*(1), 173-179.

[17] Hosseinzadeh, H.; Nassiri-Asl, M.; Avicenna's (Ibn Sina) the Canon of Medicine and saffron (*Crocus sativus*): a review. *Phytother. Res.*, **2013**, *27*(4), 475-483.

[18] Galleano, M.; Verstraeten, S.V.; Oteiza, P.I.; Fraga, C.G. Antioxidant actions of flavonoids: thermodynamic and kinetic analysis. *Arch. Biochem. Biophys.*, **2010**, *501*(1), 23-30.

[19] Cheli, Y.; Ohanna, M.; Ballotti, R.; Bertolotto, C. Fifteen-year quest for microphthalmia-associated transcription factor target genes. *Pigment. Cell. Melanoma. Res.*, **2010**, *23*, 27-40.

[20] Tada, M.; Kohno, M.; Kasai, S.; Niwano, Y. Generation mechanism of radical species by tyrosine-tyrosinase reaction. *J. Clin. Biochem. Nutr.*, **2010**, *47*(2), 162-166.

[21] Kim, M.Y.; Lee, HE.; Im, M.; Lee, Y.; Kim, C.D.; Lee, J.H.; Seo, Y.J. Effect of adenosine on melanogenesis in b16 cells and zebrafish. *Ann. Dermatol.*, **2014**, *26*(2), 209-213.

[22] Li, C.Y.; Lee, E.J.; Wu, T.S. Antityrosinase principles and constituents of the petals of *Crocus sativus*. *J. Nat. Prod.*, **2004**, *67*(3), 437-440.

[23] Li, C.Y.; Wu, T.S. Constituents of the stigmas of *Crocus sativus* and their tyrosinase inhibitory activity. *J. Nat. Prod.*, **2002**, *65*(10), 1452-1456.

[24] Melnyk, J.P.; Wang, S.; Marcone, M.F. Chemical and biological properties of the world's most expensive spice: Saffron. *Food. Res. Int.*, **2010**, *43*, 1981-1989.

[25] Abdullaev, F.I.; Riverón-Negrete, L.; Caballero-Ortega, H.; Manuel, Hernández, J.; Pérez-López, I.; Pereda-Miranda, R.; Espinosa-Aguirre, J.J.; Use of *in vitro* assays to assess the potential antigenotoxic and cytotoxic effects of saffron (*Crocus sativus* L.). *Toxicol. In Vitro*, **2003**, *17*, 731-736.

[26] Hosseinzadeh, H.; Modaghegh, MH.; Saffari, Z. *Crocus sativus* L. (Saffron) extract and its active constituents (crocin and safranal) on ischemia-reperfusion in rat skeletal muscle. *Evid. Based. Complement. Alternat. Med.*, **2009**, *6*, 343-350.

[27] Mousavi, S.H.; Tayarani, N.Z.; Parsaee, H. Protective effect of saffron extract and crocin on reactive oxygen species-mediated high glucose-induced toxicity in PC12 cells. *Cell. Mol. Neurobiol.,* 2010, *30*(2), 185-191.

[28] Serrano-Díaz, J.; Sánchez, AM.; Maggi, L., tínez-Tomé, M.; García-Diz, L.; Murcia, MA.; Alonso, GL. Increasing the applications of *Crocus sativus* flowers as natural antioxidants. *J. Food Sci.*, **2012**, *77*, C1162-C1168.

[29] Alavizadeh, SH.; Hosseinzadeh, H. Bioactivity assessment and toxicity of crocin: a comprehensive review. *Food. Chem. Toxicol.*, **2014**, *64*, 65-80.

[30] Tashakori-Sabzevar, F.; Hosseinzadeh, H.; Motamedshariaty, V.S.; Movassaghi, AR.; Mohajeri, SA. Crocetin attenuates spatial learning dysfunction and hippocampal injury in a model of vascular dementia. *Curr. Neurovasc. Res.*, **2013**, *10*, 325-334.