

Suppressive Effect of Defatted Kiwi Fruit Seed Extract on Acute Inflammation and Skin Pigmentation

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Received October 24, 2006; Accepted May 17, 2007

While kiwi fruit seeds are ingested with sarcocarps, biological activities associated with these seeds have not been examined. We evaluated the effects of the extract of defatted kiwi fruit seeds (KSE) on inflammation and melanin production. In a mouse writhing model stimulated by acetic acid, KSE (100–400 mg/kg) suppressed the increase in abdominal capillary permeability in a dose-dependent manner. KSE inhibited lipopolysaccharide-induced prostaglandin (PG) E₂ production, but not nitric oxide (NO) production from RAW264 cells. Quercitrin and kaempferol 3-O-rhamnoside, flavonol glycosides isolated from KSE, suppressed PG E₂ and NO production only at high concentrations (100 µg/mL). Hence, contribution of these compounds to the inhibitory activity of KSE appeared to be low. KSE, quercitrin, and kaempferol 3-O-rhamnoside showed inhibitory effects on tyrosinase activity and melanin production in B16 melanoma cells. However, daily oral administration of KSE (200–800 mg/kg) for 24 days did not affect melanin production induced by ultraviolet ray (UV)-B irradiation; instead, it enhanced the disappearance of pigmentation in the guinea pig skin. Consequently, KSE was found to suppress acute inflammation and enhance melanin disappearance. Although flavonol glycosides suppressed melanin production *in vitro*, their involvement in anti-inflammatory activity and melanin disappearance of KSE was not clarified.

Keywords: kiwi fruit, inflammation, quercitrin, prostaglandin, nitric oxide, melanin

Introduction

Kiwi fruit (*Actinidia chinensis* Planch.) grows naturally in the central and southern regions of China. The fruits were propagated to New Zealand at the beginning of the 20th century, and several varieties of high quality were subsequently developed. Medicinal usage of kiwi fruit has been described in Chinese materia medica for curing arthritic pain, paralysis, and hemorrhoids. However, only traditional usages of the fruits have been documented, and there is no description regarding the application of the seeds. During the chemical and biological investigations of kiwi fruit seeds extract (KSE), we isolated two flavonol glycosides, quercitrin and kaempferol 3-O-rhamnoside (Fig. 1). Quercitrin has been reported to show anti-inflammatory activities in various animal inflammation models. For instance, quercitrin suppressed GOT and GPT elevation in mouse hepatitis models induced by D-galactosamine/lipopolysaccharide (LPS) (Matsuda *et al.*, 2002). Quercitrin was also reported to act as an anti-inflammatory prodrug to release quercetin (Comalada *et al.*, 2005) and suppressed bowel inflammation by inhibiting the expression of NF-κB and inducible nitric oxide synthase (iNOS) (Camuesco *et al.*, 2004). Moreover, Sanchez de Medina *et al.* (1996, 2002) reported that quercitrin showed suppressive activity in a rat enteritis model. Quercitrin has also been reported to

inhibit tyrosinase activity that leads to melanin production in skin (Jeong and Shim, 2004). Hence, kiwi fruit seeds containing quercitrin are considered to possess inhibitory activities against inflammation and melanin production.

Long term skin exposure to ultraviolet ray (UV) -B irradiation causes various forms of skin damage such as flare (sunburn) and melanin deposition (suntan). Several mediators are involved in these skin protective reactions. Prostaglandin (PG) E₂ released from Langerhans cells, a kind of macrophage, causes dilation of dermal capillary vessel leading to erythema (Kobayashi, 2006). Selective cyclooxygenase (COX)-2 inhibitor has been demonstrated to be effective in UV-B-induced skin damage (Wilgus *et al.*, 2002). NO is also produced from Langerhans cells and keratinocytes (Chang *et al.*, 2003). NO not only contributes to skin inflammation (Gillardon *et al.*, 1995) but also stimulates melanin production (Lassalle *et al.*, 2003). A recent study shows that NO inhibitors improved the ability of sunscreen to protect skin against sunburn (Russo and Halliday, 2006). Hence, examination of PG E₂ and NO production was necessary to investigate the effects of KSE on inflammation and pigmentation. In this study, we focused on the effect of KSE and its flavonol glycosides on skin damages and evaluated the effects on inflammation and melanin production.

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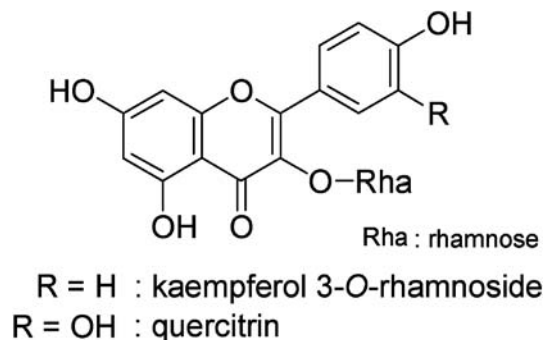


Fig. 1. Chemical Structures of Flavonol Glycosides in KSE.

Materials and Methods

Extraction of KSE and Isolation of Flavonol Glycosides

Kiwi fruit seeds, derived from Qinmei and Yate species cultivated in Shanxi province in China, were collected from Chinese beverage companies. Dried kiwi fruit seeds (1 kg) purchased from Sichuan Xiangzhen Enterprise Co., Ltd. (Chengdu, China) were mashed and defatted 3 times with *n*-hexane (60°C, 5 L) to eliminate interference of oil components for following extraction step by aqueous ethanol. After drying at room temperature, the defatted seeds were extracted (70°C, 2 h) with 70% ethanol (5 L), and then the solvent was evaporated to obtain KSE (yield: 2.1%). KSE was further purified by HPLC to obtain flavonol glycosides. KSE (20 g) was separated by preparative HPLC [column: ODS (GL Science, Tokyo, Japan, 10 ϕ mm \times 250 mm), solvent: 40% MeOH, flow rate: 2.5 mL/min] equipped with a UV (254 nm) detector. Isolated flavonols (12.9 and 4.9 mg) were identified as quercitrin and kaempferol 3-O-rhamnoside by their H^1 , C^{13} -NMR spectra (Agrawal, 1989) and MS spectra. The contents of quercitrin and kaempferol 3-O-rhamnoside in KSE determined by HPLC were 64.7 mg/100 g and 24.4 mg/100 g.

Acetic Acid-induced Mice Writhing Model Fasted (20 h) male mice (ddY, 5 to 6 weeks; Japan SLC, Inc., Shizuoka, Japan) were administered the test sample solution that was suspended with 5% w/v gum arabic in water. Fifty-five minutes after the administration, 2% pontamine sky blue dissolved in saline (10 mL/kg) was injected into the tail vein followed by injection of 1% acetic acid (10 mL/kg) into the abdominal cavity. Five minutes after the acetic acid injection, writhing behavior was counted for 15 min. Each mouse was sacrificed and the abdominal cavity was opened. The abdominal cavity was gently washed with 8 mL saline and the wash solution was filled up to 10 mL with saline. The absorbance of the wash solution at 590 nm was measured, and the amount of leaked blue dye was calculated from the standard curve plotted by a series of dilutions of standard pontamine sky blue solution.

LPS-induced PG E_2 and NO Production from RAW264 Cells RAW264 cells (1×10^6 cells/mL; RIKEN Bioresource Center, Tsukuba, Japan), mouse leukemic monocytes, suspended in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% fetal calf serum (FCS, Invitrogen Canada, Ontario,

Canada), 100 units/mL penicillin G, and 100 μ g/mL streptomycin were seeded (200 μ L/well) in a 48-well culture plate and pre-cultured for 24 h. LPS (final concentration: 10 μ g/mL, from *E. coli*, Serotype 0127: B8; Sigma-Aldrich, St. Louis, MO, USA) and the test sample solution were added to each well and cultured for 20 h. Indomethacine (Sigma-Aldrich) was used as a positive control. The supernatant was collected and stored at -80°C . PG E_2 content in the supernatant was determined using a commercial kit (Prostaglandin E_2 EIA Kit-Monoclonal, Cayman Chemical Corporate, Ann Arbor, MI, USA) and NO content was determined by the Griess method (Griess reagent kit; Dojin Chemical, Kumamoto, Japan).

Examination of Tyrosinase Inhibitory Activity The test sample solution (70 μ L) was mixed with an equal volume of 0.3% L-DOPA (Sigma-Aldrich) and incubated at 37°C for 5 min. Tyrosinase (70 μ L, 1.6 units/mL, Sigma-Aldrich) derived from mushrooms was added to the test solution and incubated for 5 min. After incubation, the absorbance was measured at 492 nm.

Melanin Production in B16 Melanoma B16 melanoma (5×10^4 cells/mL; Japan Health Science Foundation, Osaka, Japan), mouse skin melanoma cell lines, in α -modified Eagle's medium containing 10% FCS, 100 units/mL penicillin G, 100 μ g/mL streptomycin, and 2 mM theophylline were seeded in a 48-well culture plate at 200 μ L/well. After 24 h, each sample was added to the culture plates, and the cells were cultured for 3 days. The medium was gently removed, and the cells were sonicated in 200 μ L/well of phosphate buffered saline. The absorbance of each well was measured at 415 nm.

UV-B-induced Melanin Production in Guinea Pig Skin Male guinea pigs (Weiser-Maples, 4 weeks old; Kiwa Research Laboratory, Wakayama, Japan) were orally administered KSE once a day for 7 days prior to UV-B irradiation (day -7). The shaved back skin of the guinea pigs was then irradiated with UV-B (2000 mJ/cm 2) using a UV-ray radiator (Solar simulator type X 150; Ushio, Inc., Tokyo, Japan) for 7 days (days 0–6). KSE was given to the guinea pigs during the days for UV-B irradiation and continuously for 10 days after the irradiation (days 0–16). The brightness intensity (L^* value) of the irradiated spot on the back skin was measured using a spectro color meter (SE 2000; Nippon Denshoku Industries Co., Ltd., Tokyo, Japan) as an indicator of the clearance of the pigmentation.

Statistics The results are shown as mean \pm S.E. Dunnett's multiple range test was used for the evaluation of significance. $P < 0.05$ is considered to indicate statistical significance.

Results and Discussion

Anti-inflammatory Effects of KSE In acetic acid-induced mouse writhing model, an acute inflammation model, KSE (100–400 mg/kg) slightly suppressed the writhing behavior (Fig. 2). However, the effects were not significant. In contrast, KSE (100–400 mg/kg) significantly ($p < 0.05$) suppressed dye leakage due to the increase in permeability of the abdominal capillaries. The maximal inhibi-

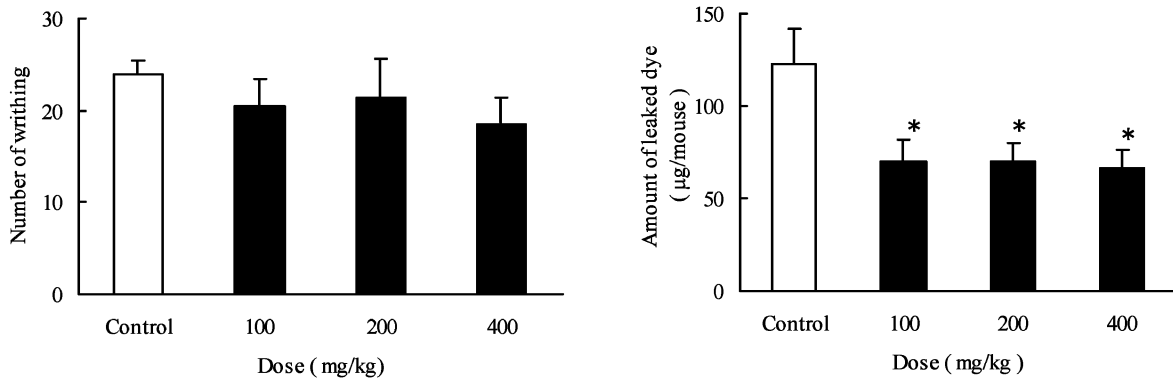


Fig. 2. Analgesic and Anti-inflammatory Effects of KSE on Acetic Acid-induced Mouse Writhing Model. Each column represents mean and S.E. ($n=11$). An asterisk denotes significant difference from control *: $p < 0.05$.

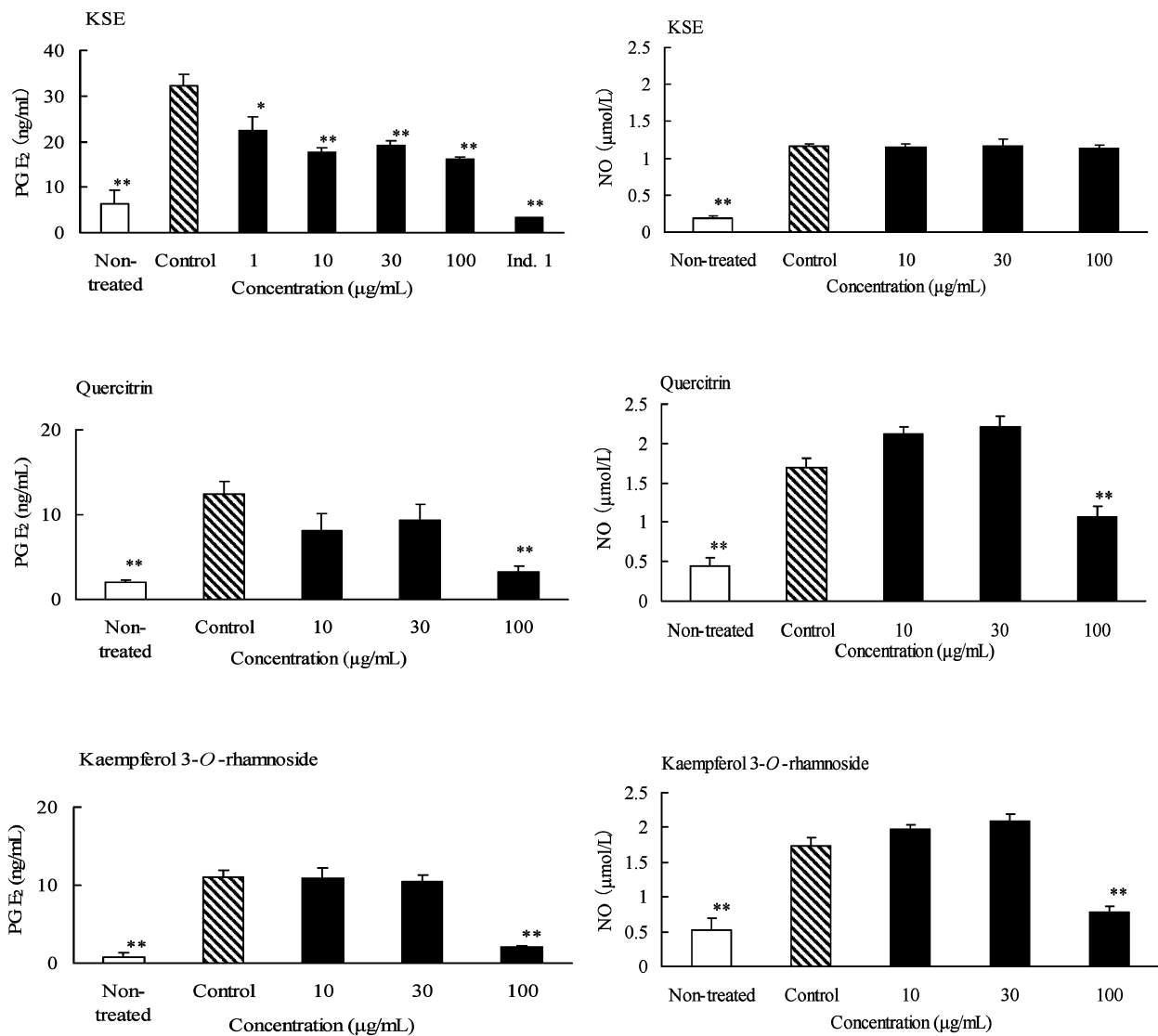
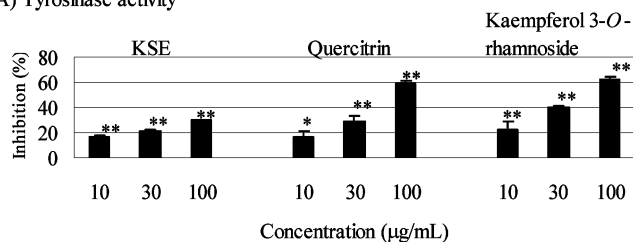


Fig. 3. Effects of KSE and Flavonol Glycosides on PG E₂ and NO Production from RAW264 cells Induced by LPS. Each column represents mean with the S.E. ($n=6$). Asterisks denote significant differences from control. *: $p < 0.05$, **: $p < 0.01$, respectively. Ind : indomethacin.

A) Tyrosinase activity



B) Melanin production

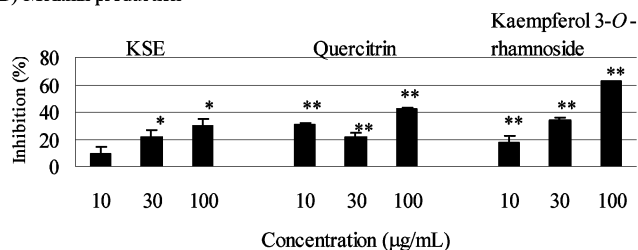


Fig. 4. Inhibitory Effects of KSE and its Flavonol Glycosides on Tyrosinase Activity and Melanin Production from B16 Melanoma.

Each column represents mean with the S.E. ($n=3$). Asterisks denote significant differences from the non-sample-treated group. * : $p < 0.05$, ** : $p < 0.01$, respectively.

tion was 45.6% at 400 mg/kg. These results indicate that KSE possesses anti-inflammatory activity by oral administration.

To evaluate the anti-inflammatory property of KSE and involvement of the flavonol glycosides, we continuously evaluated the effects of KSE and flavonol glycosides on PG E_2 and NO production from LPS-stimulated RAW264 cells. By LPS (10 $\mu\text{g}/\text{mL}$) stimulation, PG E_2 and NO production were significantly ($p < 0.01$) increased more than 5.0 and 3.2 fold higher than LPS-untreated cells, respectively. KSE (1–100 $\mu\text{g}/\text{mL}$) significantly (1 $\mu\text{g}/\text{mL}$: $p < 0.05$, >10 $\mu\text{g}/\text{mL}$: $p < 0.01$) suppressed PG E_2 production from RAW264 cells (Fig. 3). The maximal inhibition was 49.8% at 100 $\mu\text{g}/\text{mL}$. However, KSE did not suppress NO production under identical conditions. High concentrations (100 $\mu\text{g}/\text{mL}$) of quercitrin and kaempferol 3-*O*-rhamnoside significantly ($p < 0.01$) suppressed PG E_2 and NO production. However, both compounds had no effect on PG E_2 and NO production at concentrations less than 30 $\mu\text{g}/\text{mL}$. These results indicate that KSE suppresses PG E_2 production from RAW264 cells and has no effect on NO production. Flavonol glycosides are not considered to be involved in the suppressive effect of KSE on PG E_2 production too much, because these compounds showed inhibitory effects only at high concentrations (100 $\mu\text{g}/\text{mL}$). In a preliminary examined cytotoxicity test guided by formazan formation in mitochondria, KSE and flavonol glycosides did not interfere with cell growth up to 100 $\mu\text{g}/\text{mL}$. Other unknown constituents may suppress PG E_2 production through inhibition of cyclooxygenase activity. Moreover, there remains the possibility that these constituents may affect other processes of inflammation such as increase in capillary permeability and production of other in-

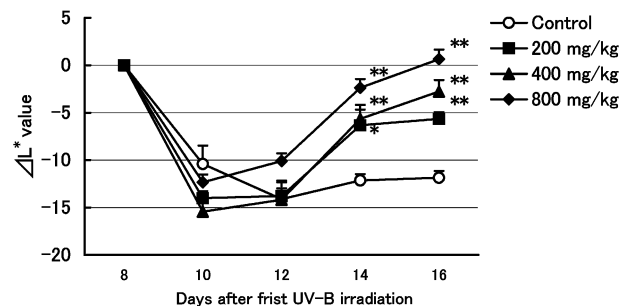


Fig. 5. Effect of KSE on Skin Hyperpigmentation in Guinea Pigs Induced by UV-B.

Each point represents mean with the S.E. ($n=4$). Asterisks denote significant differences from control. * : $p < 0.05$, ** : $p < 0.01$, respectively.

flammatory mediators. Further investigation is required to identify the inhibitors of PG E_2 and NO production in KSE and to clarify the involvement of flavonol glycosides in other anti-inflammatory mechanisms.

Inhibitory Effect of KSE and Flavonol Glycosides on Melanin Production To examine the effect of KSE on melanin production, two *in vitro* assays were chosen. KSE and flavonol glycosides inhibited both tyrosinase activity and melanin production from B16 melanoma at concentrations of 10 to 100 $\mu\text{g}/\text{mL}$ (Fig. 4). Inhibitory effects of flavonol glycosides on both melanin production models were well correlated with those of KSE. These results show that the inhibitory effect of KSE on melanin production from melanoma is partially based on tyrosinase inhibitory activity. Moreover, flavonol glycosides were confirmed to act as inhibitors of melanin production in KSE.

Based on reliable results of *in vitro* examination, we evaluated the effect of KSE on melanin production in the skin of guinea pigs induced by UV-B irradiation. The L^* value that indicates difference of brightness was used as an indicator of melanin production. Decrease in L^* value reveals a change in color to darker. The change in color was observed after 8 days. On days 10 and 12 after the start of UV-B irradiation, L^* values were lower than those of day 8 (Fig. 5). KSE did not affect the formation of pigmentation on days 10 and 12. However, KSE (200–800 mg/kg) enhanced the recovery of L^* value from days 14 to 16 in a concentration-dependent manner, contrast to little change in L^* value of control. The result shows that KSE does not affect melanin production, but enhance the disappearance of produced melanin. KSE possibly enhances skin metabolism cycle or peripheral blood circulation leading to melanin disappearance. The *in vitro* study showed that flavonol glycosides in KSE inhibited melanin production. However, these compounds do not seem to act *in vivo*, and their participation in melanin decomposition was not clarified. Further studies are required to examine the effects of flavonol glycosides on the process of melanin decomposition.

In recent years, many beneficial effects of kiwi fruit consumption have been reported. For example, Kuriyama *et al.* found that frequent intake of kiwi fruits lowered

plasma 8-isoprostaglandin $F_{2\alpha}$, a lipid peroxidation biomarker, in elderly Japanese subjects (Kuriyama *et al.*, 2006). Duttaroy and Jorgensen reported that platelet aggregation response and blood triglyceride in human volunteers were improved by daily intake of two or three kiwi fruits for 28 days (Duttaroy and Jorgensen, 2004). Kiwi fruit is well known to contain a large amount of vitamin C (Szeto *et al.*, 2002) and both authors previously suggested the contribution of vitamin C to the beneficial effects of kiwi fruit described above. The edible part of kiwi fruits contain lignins (Bunzel and Ralph, 2006), proteins including an allergen (Tamburrini *et al.*, 2005) and a pectin methylesterase (Ciardiello *et al.*, 2004), and polysaccharides that enhance collagen synthesis in keratinocytes (Deters *et al.*, 2005). Moreover, actinidic acid, a new triterpene, and other triterpenes have been isolated from the peel of unripe kiwi fruit (Lahlou *et al.*, 2001). Although several observations have been reported in recent years, no ingredients or biological activities of kiwi fruit seed have been examined so far. In this study we prepared an aqueous ethanol extract from defatted kiwi fruit seeds and examined its constituents and biological activities. As a result, quercitrin and kaempferol 3-*O*-rhamnoside were isolated as polyphenolic compounds in the extract. Hence, we evaluated the biological activity of KSE and found the anti-inflammatory activity and the pigmentation clearing effect in *in vivo* models. Although quercitrin and kaempferol 3-*O*-rhamnoside were not found to be involved in the inhibitory effect of KSE on PG E_2 and NO production from macrophages, these compounds were confirmed to suppress melanin formation in melanoma cells. However, the compounds do not seem to suppress melanin production in an *in vivo* model. In conclusion, kiwi fruit seed is demonstrated to possess beneficial effects against inflammation and pigmentation.

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