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Inhibitory Effect of an Ellagic Acid-Rich Pomegranate Extract on Tyrosinase Activity and Ultraviolet-Induced Pigmentation

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A pomegranate extract (PE) from the rind containing 90% ellagic acid was tested for its skin-whitening effect. PE showed inhibitory activity against mushroom tyrosinase *in vitro*, and the inhibition by the extract was comparable to that of arbutin, which is a known whitening agent. PE, when administered orally, also inhibited UV-induced skin pigmentation on the back of brownish guinea pigs. The intensity of the skin-whitening effect was similar between guinea pigs fed with PE and those fed with L-ascorbic acid. PE reduced the number of DOPA-positive melanocytes in the epidermis of UV-irradiated guinea pigs, but L-ascorbic acid did not. These results suggest that the skin-whitening effect of PE was probably due to inhibition of the proliferation of melanocytes and melanin synthesis by tyrosinase in melanocytes. PE, when taken orally, may be used as an effective whitening agent for the skin.

Key words: pomegranate extract; ellagic acid; tyrosinase; pigmentation; whitening

The pomegranate (*Punica granatum* L.) has been extensively used in traditional medicines in many countries. The Chinese, for example, have used pomegranate as a traditional product in antibacterial, anti-inflammatory, and hemostasis applications. Extracts from different parts of this plant such as the juice,[1,2] seed,[3] and peel[4] have been reported to exhibit strong antioxidative activity. Pomegranate juice has a potent antiatherogenic effect in humans and in atherosclerotic mice, which may be attributable to its antioxidative properties.[2,5] Dry pomegranate seed contains the steroid, estrogen estron, the isoflavone phytoestrogens, genistein and daidzein, the phytoestrogen, coumestrol,[6] and other compounds.

Pomegranate is rich in phenolic compounds, and is an important source of anthocyanins, 3-glucosides, and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin.[7] In addition, pomegranate bark is very rich in ellagitannins and gallotannins.[8] Ellagic acid ($C_{14}H_6O_8$, Fig. 1) is a naturally occurring phenolic compound found in many natural sources, some common ones being strawberries, raspberries, blackberries, and pomegranates. Ellagitannins are also found in various plants and hydrolyzed to ellagic acid under acidic conditions. Ellagic acid has been found to have anticarcinogenic,[9,10] antifibrosis,[11] and antioxidative[12] properties. It has been reported that ellagic acid has a high affinity for copper at the active site of tyrosinase and inhibits its activity.[13] Shimogaki *et al.* have reported that when ellagic acid was topically applied, it suppressed UV-induced skin pigmentation of brownish guinea pigs.[13]

However, relatively little is known about the whitening activity of ellagic acid or of a pomegranate extract rich in ellagic acid when administered orally. In the present study, a pomegranate extract rich in ellagic acid (90%) was prepared, and its inhibitory activity toward tyrosinase was investigated *in vitro*, together with the whitening effect on UV-induced pigmentation of brownish guinea pig skin by an oral administration.

Materials and Methods

Pomegranate extract. Pomegranate (*Punica granatum* L.) fruit rind was extracted 3 times with 50% aqueous ethyl alcohol at 60 °C–70 °C for 2 hours. The ethyl alcohol was removed under vacuum. The resulting aqueous solution was acidified with hydrochloric acid and then refluxed at 70 °C for 6 hours. Upon dilution with water, ellagic acid was precipitated. The precipitate was collected by filtration and dried in a vacuum tray drier. This pomegranate extract (PE) contained 90.16% ellagic acid on a dry basis (confirmed by an HPLC analysis). This extract was used for the tyrosinase inhibition assay and the animal study.

Materials. L-Tyrosine, L-(+)-ascorbic acid, sodium dihydrogen phosphate, disodium hydrogen phosphate and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemicals (Osaka, Japan). Arbutin was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Mushroom tyrosinase was purchased from Calzyme

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Abbreviation: PE, pomegranate extract

(CA, U.S.A.), and L-3,4-dihydroxyphenylalanine (DOPA) was purchased from Sigma Chemicals (MO, U.S.A.).

Tyrosinase inhibition assay using mushroom tyrosinase. A tyrosinase inhibition assay was performed according to the procedure of Lee *et al.*[14] with slight modifications. Fifty μl of L-tyrosine (2 mM), 90 μl of a 0.1 M phosphate buffer (pH 6.8) and 10 μl of DMSO with or without a sample were added to a 96-well microplate. The plate was preincubated at 37 °C for 5 minutes, before 50 μl of a phosphate buffer with or without mushroom tyrosinase (0.125 mg/ml) was added. After incubating at 37 °C for 15 minutes, the amount of DOPAchrome was determined at 405 nm. The percentage inhibition of tyrosinase activity was calculated as the inhibition (%) = [(A–B)/A] × 100, where A represents the difference in absorbance of the control sample between the samples with and without tyrosinase, and B represents the difference in the test sample between the samples with and without tyrosinase.

Animal study on guinea pigs with UV-induced pigmentation. The inhibitory effect on UV-induced pigmentation was investigated by using brownish guinea pigs. Female brownish guinea pigs, Kwl:A-1 with a body weight of 246–356 g, were purchased from Kiwa Laboratory Animals Co. (Wakayama, Japan). All animals had free access to food and water and were kept in an air-conditioned room (20 °C–26 °C, 40%–70% humidity) under a 12 h dark/light cycle. During the experimental period, the guinea pigs received humane care consistent with institutional guidelines. Six animals per group were used. The back of each brownish guinea pig was cleanly shaved with electric clippers. A 4-cm^2 area on the shaven skin was irradiated with 64.8 J/cm^2 (0.09 mW/cm^2 × 12 min) from an ultraviolet (UV) B lamp (Toshiba FL20S E, Tokyo, Japan). The guinea pigs were divided into 4 groups and orally administered with water, PE or L-ascorbic acid for 35 days as follows: group 1 as the control received only water, group 2 received 100 mg/kg/day of PE diluted in water at 10 mg/ml, group 3 received 1,000 mg/kg/day of PE diluted in water at 100 mg/ml, and group 4 received 600 mg/kg/day of L-ascorbic acid diluted in water at 60 mg/ml.

The guinea pigs were irradiated on days 7, 9, and 11 as just described. The whitening effect was determined by measuring the L* value weekly with a reflectance spectrophotometer (Minolta CR-300, Tokyo, Japan). The blanching effect was quantified by the increase in L*-value:

$$\Delta L^* = L^* \text{ (on the measuring day)}$$
$$- L^* \text{ (on the first day of the test,}$$
$$\text{before UV-irradiation)}$$

After the test period, on day 36, the animals were sacrificed according to institutional guidelines. The back skin samples were collected and stored at −80 °C.

Measurement of DOPA-positive melanocytes in guinea pigs. The skin tissue samples were taken from UV-irradiated and untreated areas of the tested guinea pigs, and DOPA-staining of the epidermal sheet was performed by the method of Staricco *et al.*[15] The number and size of DOPA-positive melanocytes were measured under an optical microscope. The size of the melanocytes was measured across the cytoplasm without dendrites.

Statistical analysis. Each data value is expressed as the mean +/− SEM. One-way ANOVA with Dunnett's significant difference test was also used to evaluate differences among the groups.

Result

The tyrosinase inhibitory activity of PE was examined and compared with that of L-ascorbic acid and arbutin, which are known tyrosinase inhibitors. PE inhibited mushroom tyrosinase activity with an IC$_{50}$ value of 182.2 μg/ml. This inhibitory activity of PE is comparable to that of arbutin, but was about ten times weaker than that of L-ascorbic acid (Table 1), indicating that PE is a tyrosinase inhibitor.

Further studies on the *in vivo* inhibitory effect of PE on UV-induced pigmentation were performed. The general condition and behavior of all rats were normal. The body weight and food consumption of animals in all groups during the course of this study were almost the same. Figure 2 shows photographs of typical skin samples after the oral administration of PE, L-ascorbic acid or water as a control. The UV-induced skin pigmentation was reduced in the PE or L-ascorbic acid group, but not in the control group. A quantitative evaluation of whitening was done by determining the rate of change of L* value after 5 weeks of admin-
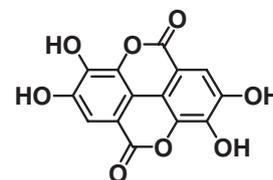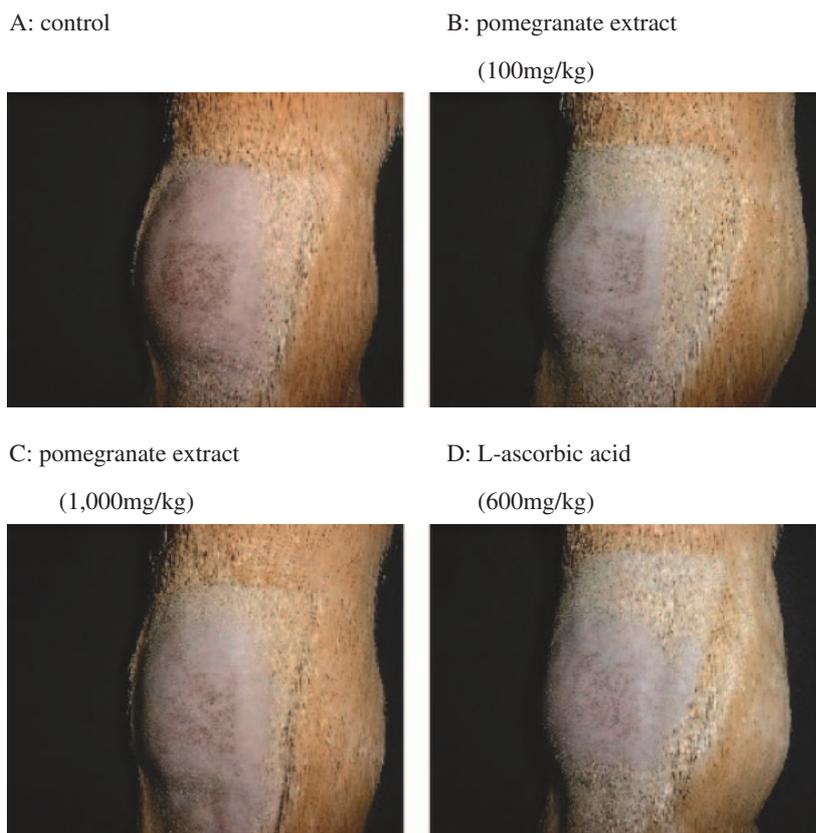

Fig. 1. Structure of Ellagic Acid.

Table 1. Tyrosinase Inhibitory Activity

Test sample	IC$_{50}$ (μg/ml)
Pomegranate Extract	182.2
Arbutin	162.2
L-Ascorbic Acid	18.4

A: control

B: pomegranate extract

(100mg/kg)

C: pomegranate extract

(1,000mg/kg)

D: L-ascorbic acid

(600mg/kg)

Fig. 2. Lightening Effects of the Samples on UV-Induced Hyperpigmentation.

Representative photographs show the lightening effects of the samples (A, control; B, pomegranate extract (100 mg/kg); C, pomegranate extract (1,000 mg/kg); D, L-ascorbic acid (600 mg/kg)) on UV-induced hyperpigmentation after 35 days of administration (n = 6). These photographs show typical skin from each group.

A: control

B: pomegranate extract (1000mg/kg)
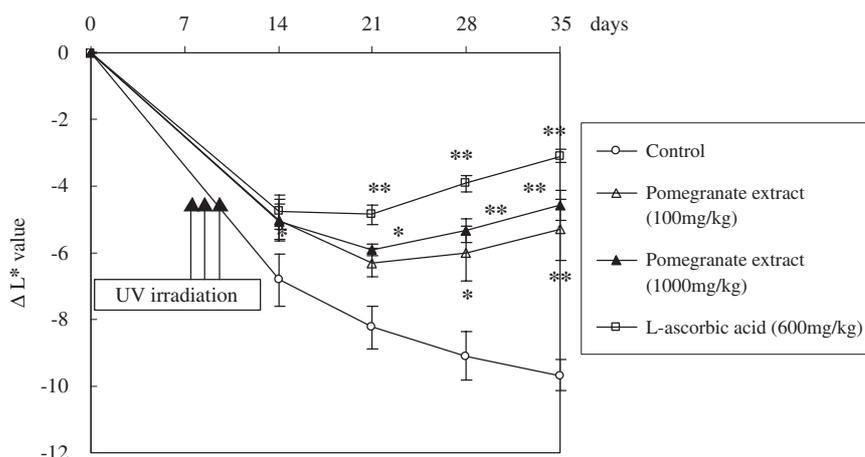

C: L-ascorbic acid (600mg/kg)
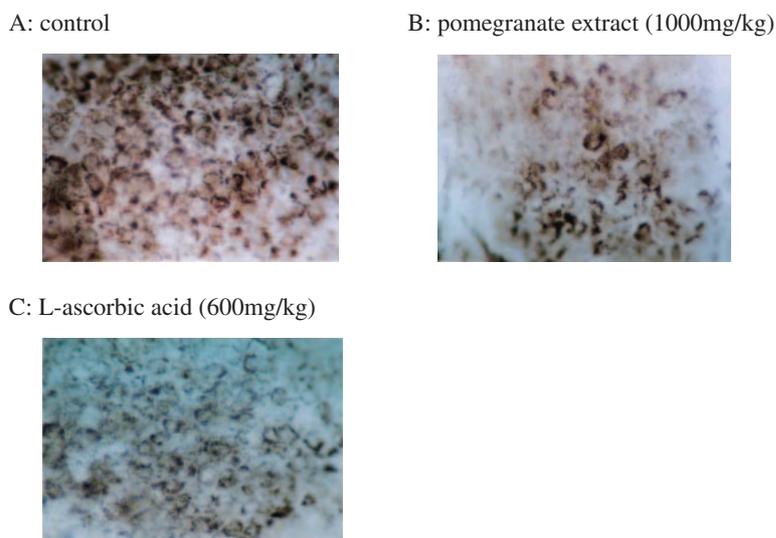
Fig. 4. DOPA-Positive Melanocytes in the Epidermis of UV-Irradiated Skin.

Photographs are shown of DOPA-positive melanocytes in the epidermal sheet of the UV-irradiated skin of brownish guinea pigs administered with each sample for 35 days. A, water (control); B, pomegranate extract (1,000 mg/kg); C, L-ascorbic acid (600 mg/kg) (n = 6). These photographs show typical skin samples from each group.

istration of these samples. As shown in Fig. 3, hyperpigmentation was weakened more effectively by the administration of both doses of PE or L-ascorbic acid

than in the control case. PE showed a whitening effect in a dose-dependent manner. The ΔL^* value was not statistically different among both PE groups and the L-

Fig. 3. Degree of Pigmentation.

The degree of pigmentation change (ΔL^* value) is shown between the start, after UV irradiation and after daily administration of water (control, circle), the pomegranate extract (100 mg/kg, unshaded triangle), pomegranate extract (1,000 mg/kg, shaded triangle), and L-ascorbic acid 600 mg/kg (square) (n = 6).
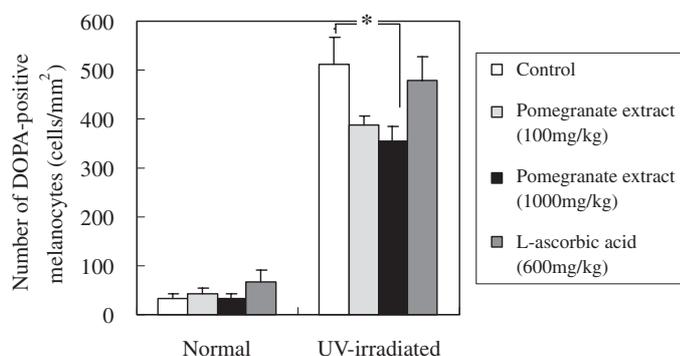
Fig. 5. Number of DOPA-Positive Melanocytes.

The number of DOPA-positive melanocytes in the epidermis of guinea pigs administered with water (control), the pomegranate extract, and L-ascorbic acid for 35 days is shown (n = 6). *, $p < 0.05$ compared with the control.
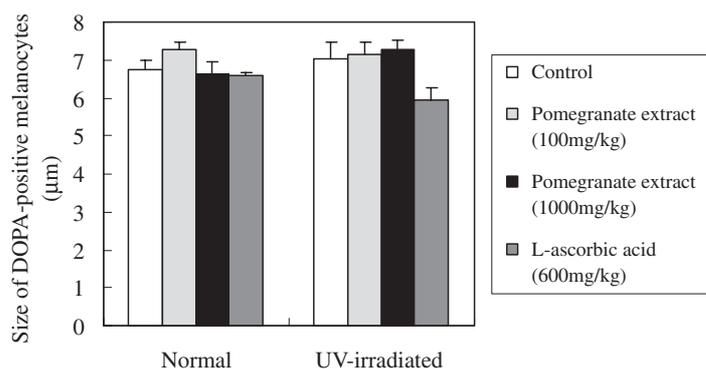
ascorbic acid group.

Figure 4 shows photographs of the DOPA-positive melanocytes in the epidermal sheet of the UV-irradiated skin of brownish guinea pigs. DOPA-positive melanocytes with many dendrites were sparse in the untreated area of all groups (data not shown). In contrast, in the UV-irradiated areas of the control group, a large number of melanocytes formed a dense network (Fig. 4A). In the PE group (1000 mg/kg), the number of melanocytes was lower than in the control group (Fig. 4B). In the L-ascorbic acid group, the number of melanocytes was similar to the number observed in the control group. However, the intensity of DOPA staining in the guinea pigs administered with L-ascorbic acid was considerably weaker than that in the control group (Fig. 4C).

Figure 5 shows a quantitative measurement of the number of DOPA-positive melanocytes. The number of melanocytes in the UV-irradiated skin of the high-dose PE group (1000 mg/kg) was significantly lower than in the control group in a dose-dependent manner (Fig. 5). In contrast, the number of melanocytes in the L-ascorbic

acid group was not reduced (Fig. 5). The size of DOPA-positive melanocytes in the both PE groups was comparable to that of the control group (Fig. 6). There was no difference in the number and size of DOPA-positive melanocytes in the normal skin of all groups (Fig. 5 and 6).

Discussion

The synthesis and activation of tyrosinase are major processes in the UV-induced pigmentation of mammals.[16] Many tyrosinase inhibitors such as proanthocyanidin,[17] arbutin[18] and ellagic acid[13] have been isolated from plants. Several of these tyrosinase inhibitors have been used in cosmetics for topical application, but few are used for oral administration. Oral administration of ellagic acid has been studied in the metabolism of the rat, and its metabolites have been detected in their urine and feces.[19] We hypothesized from these results that ellagic acid might have a whitening effect if administered orally. To obtain a

Fig. 6. Size of DOPA-Positive Melanocytes.

The size of DOPA-positive melanocytes in the epidermis of guinea pigs administered with water (control), the pomegranate extract, and L-ascorbic acid for 35 days is shown (n = 6). No significant difference was apparent.

natural ellagic acid preparation, pomegranate fruit rind was extracted with 50% aqueous ethyl alcohol followed by acid hydrolysis. We demonstrate in this study that the pomegranate extract (PE), when taken orally, had an inhibitory effect on tyrosinase *in vitro* and a whitening effect *in vivo* on UV-induced pigmentation of brownish guinea pig skin.

PE inhibited mushroom tyrosinase activity *in vitro*, but the inhibitory activity was about ten times weaker than that of L-ascorbic acid. When PE was administered orally to brownish guinea pigs, a whitening effect on UV-induced pigmentation, comparable to that of L-ascorbic acid, was observed. PE also reduced the number of DOPA-positive melanocytes on UV-induced pigmentation without any effect on the size of these melanocytes. Conversely, L-ascorbic acid did not affect the number of DOPA-positive melanocytes, but especially weakened the DOPA staining density of the melanocytes.

Quevedo *et al.*[20] have reported that the UV radiation-induced proliferation and melanogenesis of melanocytes was reduced by a topical application of an antioxidant such as vitamin C or E to the skin of hairless mice. UV irradiation induced 8-OHdG (a representative DNA base-modified product generated by reactive oxygen species [ROS]) within DNA of cultured mouse keratinocytes,[21] and also induced the proliferation of keratinocytes in human skin.[22] Therefore, ROS are considered to play an important role in regulating the proliferation of melanocytes and keratinocytes, and the melanogenesis of melanocytes. Ellagic acid is a ROS scavenger and has potent antioxidative activity, resulting in an inhibition of peroxidation in the skin.[23] Such antioxidative and ROS-scavenging activities of ellagic acid may contribute to the lightening effect of PE on the UV-induced pigmentation of brownish guinea pigs. On the other hand, L-ascorbic acid prevents melanin formation, according to the mechanism for the inhibition of tyrosinase and the reduction of DOPAquinone to DOPA.[24] L-ascorbic acid could reduce the melanin concentration of melanoma cells.[25] Our results seem to

indicate that PE had different effects from L-ascorbic acid for anti-pigmentation, although it was not clear by this examination what the difference of correct mechanism involved.

The pharmacokinetic properties of ellagic acid have been studied.[26] When a pomegranate leaf extract containing 85.3 mg/kg of ellagic acid was orally administered, most of the ellagic acid was absorbed in the stomach and rapidly eliminated.[26] The antioxidative activity and antiatherogenic effect of orally administered pomegranate juice have been reported.[1,2] In addition, Mukhtar *et al.* have reported that orally administered ellagic acid inhibited skin tumorigenicity in mice.[9] It might therefore be possible for ellagic acid and/or its antioxidative metabolites in our pomegranate extract to be absorbed and distributed to the skin by an oral administration. We found that orally administered PE containing 90% ellagic acid inhibited UV-irradiated pigmentation on brownish guinea pig skin, and suggest that PE had a whitening effect on the skin from the oral administration. Accordingly, absorbed ellagic acid and/or its antioxidative metabolites may have contributed to the whitening effect on the pigmented skin of brownish guinea pigs.

In conclusion, an oral administration of PE effectively whitened the pigmented skin of guinea pigs. This effect was probably due to inhibition of the proliferation of melanocytes and melanin synthesis by tyrosinase in melanocytes. PE, when taken orally, may be a useful skin-whitening agent.

Acknowledgment

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