Studies of Cuticle Drugs from Natural Sources. II. Inhibitory Effects of *Prunus* Plants on Melanin Biosynthesis

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The inhibitory effects of 50% ethanolic extracts from dried leaves of 38 plants collected in the herbal garden of Kinki University were investigated *in vitro* on melanin biosynthesis which is closely related to hyperpigmentation. Of the 38 extracts, *Prunus yedoensis*, *P. zippeliana*, *P. amygdalus*, *P. persica*, *P. armeniaca*, *Thea sinensis* and *Chaenomeles sinensis* showed a potent inhibition of tyrosinase, the enzyme which converts 3-(3,4-dihydroxyphenyl)alanine (dopa) to dopachrome in the biosynthetic process. Furthermore, the extracts from the leaves of *P. yedoensis* and *P. zippeliana* among the *Prunus* plants used in this experiment inhibited the production of melanin from dopachrome by autoxidation. These inhibitory effects of *P. zippeliana* on melanin biosynthesis were observed in cultured B-16 mouse melanoma cells.

These results suggest that the leaves of *P. zippeliana* inhibit melanin biosynthesis which is involved in hyperpigmentation and could be used as a whitening agent for the skin.

Keywords *Prunus zippeliana*; melanin; tyrosinase; B-16 mouse melanoma cell; hyperpigmentation; whitening agent

Recently, the application of crude drugs in cosmetics has generated keen interest. We reported that crude drugs originating from Umbelliferous plants, which were applied as cosmetic beauty preparations, have an inhibitory effect on tyrosinase activity which is closely related to hyperpigmentation.¹⁾ In the present study, the leaves of 38 plants collected in the herbal garden of Kinki University and 4 crude drugs originating from the *Prunus* plant were investigated *in vitro* for the action on a process involved in melanin biosynthesis.

MATERIALS AND METHODS

Materials The thirty-eight plants shown in Table I were collected in the herbal garden of Kinki University (Osaka, Japan) and the dried leaves were used in the experiments. Persicae Semen, Armeniacae Semen, Pruni Cortex and sweet almond were obtained from Nippon Funmatsu Yakuhin Co. Ltd. (Japan). The 50% ethanolic extracts from these leaves and crude drugs were prepared as follows: First, the crude leaves were dried in the shade. The dried leaves or crude drugs were then formed to pieces and extracted with ten volumes (w/v) of 50% ethanol at about 20 °C for 5 d; the extracts were obtained by evaporation and freeze-drying. In addition, benzaldehyde (Kishida Chemicals) contained in the leaves of *P. zippeliana* was used as a test substance. The following drugs were also used in this study: kojic acid, 3-(3,4-dihydroxyphenyl)-L-alanine (L-dopa), deoxycholic acid sodium salt and trichloroacetic acid (Nakalai Tesque), tyrosinase (from mushroom, Sigma Chemical Co.), melanin (Sigma Chemical Co.) and solucene 350 (Canberra Co.).

Tyrosinase Activity Tyrosinase activity was measured according to the method of Mason and Peterson.²⁾ First, test substances dissolved 0.5 ml 1/15 M phosphate buffer (pH 6.8) were incubated at 25 °C for 10 min, and then 0.5 ml mushroom tyrosinase (135 U/ml) and 0.5 ml 0.03% dopa solution were added to the solution and incubated

for 5 min. The amount of dopachrome in the reaction mixture was determined using a Hitachi spectrophotometer, model 200-10, measuring the optical density (OD) at 475 nm. Kojic acid was used as a standard. The percentage inhibition of tyrosinase was calculated as follows;

$$\% \text{ inhibition} = [(A - B) - (C - D)] / (A - B) \times 100$$

A: OD at 475 nm without test substance

B: OD at 475 nm without test substance and enzyme

C: OD at 475 nm with test substance

D: OD at 475 nm with test substance, but without enzyme

Production of Melanin from Dopachrome by Autoxidation Production of melanin from dopachrome by autoxidation was measured by the modified method of Mason and Peterson.²⁾ After incubation of the tyrosinase solution at 25 °C for 10 min, dopa solution was added and incubated for 5 min. Test substances were then added to the solution and incubated for 60 min. The reaction was stopped by adding 0.2 ml 1 N HCl and the mixture was centrifuged at 3000 rpm for 15 min. The precipitate obtained was washed with 1 ml 6 N HCl, distilled water (twice), and dissolved in 2 ml solucene 350.³⁾ The OD of the solutions were determined at 400 nm. The amounts of melanin were expressed as the product of the OD at 400 nm and the volume of the solution; the weight of standard melanin and the percentage inhibition of the test substances were determined. Kojic acid was used as a standard.

Tyrosinase Activity and Melanin Content in Cultured B-16 Melanoma Cells The tyrosinase activity and melanin content in cultured B-16 melanoma cells were measured by the method of Sato *et al.*⁴⁾ All cell lines were cultured in Eagle's minimum essential medium (MEM, Nissui Seiyaku Co.) supplemented with 10% calf serum. The medium was changed every 2 or 3 d. Cells were detached with trypsin-EDTA solution (0.05% trypsin, 0.53 mM EDTA · 4Na, GIBCO Lab.) for subculture about

once a week, at confluency. For the experimental procedure, we grew cells as monolayer cultures in a 100 mm plastic Petri dish incubated at 37 °C in a humidified incubator in 5% CO₂–95% air.

For the experiment involving the melanogenic ability of the test substances, we changed the culture medium of both the control and experimental groups 1 d after the addition of the test substances. We supplement the medium of the experimental group with test substances (125, 250 or 500 µg/ml) from day 1 to the end of culture (3 d). The cell numbers in duplicate dishes were counted every day.

Cells were harvested with 0.05% trypsin–0.53 mM EDTA solution, then suspended in saline for cell counting. The suspension was divided into two test-tubes, one for the measurement of tyrosinase activity and the other for melanin content. The cells were washed by centrifugation at 3000 rpm for 30 min and the cell pellets were kept in 1 ml 0.5% deoxycholic acid sodium salt solution for 20 min at 0–4 °C, followed by stirring. This cell lysate was used for the assay of tyrosinase activity. Enzyme activity

was assayed spectrophotometrically at 475 nm following the oxidation of L-dopa to dopachrome.⁵⁾ The reaction mixture contained 0.3 ml of cell lysate and 3 ml 0.04% L-dopa in 0.1 M sodium phosphate buffer, pH 6.8, according to the modified method of Wrathall *et al.*⁶⁾ The dopa solution was incubated for 10 min at 37 °C before mixing. The change in OD at 475 nm was measured during the first few minutes while it was linear. Correction for dopa autoxidation was performed. Specific activity was expressed as the Δ OD at 475 nm per min per mg protein. Protein content was estimated by the method of Lowry *et al.*⁷⁾ with a standard protein solution of bovine serum (fraction V, Wako).

The melanin content was determined by the method of Whittaker.⁸⁾ The cell pellet was suspended in 1 ml distilled water and underwent three extractions with 5% trichloroacetic acid, two extractions with ether–alcohol (1:3) and one extraction with absolute ether. The air-dried residue was then dissolved in 5 ml 1 N NaOH and heated for 10 min at 100 °C. After being cooled, the OD of the

TABLE I. Inhibitory Effects on Tyrosinase Activity of 50% Ethanolic Extracts from Leaves of 38 Plants Collected in the Herbal Garden on Kinki University

Plant name	Extract yield (%)	Inhibition (%)			
		50	200	500	1000 (µg/ml)
<i>Prunus yedoensis</i> MATSUM.	24.6	31.0	62.1	80.6	
<i>Prunus zippeliana</i> MIQ.	28.5	23.5	60.0	79.3	
<i>Thea sinensis</i> L.	30.0	13.7	25.7	51.5	
<i>Chaenomeles sinensis</i> KOEHNE.	20.1	14.4	33.5	49.2	
<i>Prunus amygdalus</i> STOKES	17.9	8.6	23.3	43.0	
<i>Prunus persica</i> (L.) BATSCH	11.2	9.4	24.1	42.6	
<i>Prunus armeniaca</i> L. var. <i>ansu</i> MAXIM.	39.5	14.7	31.3	42.1	
<i>Citrus unshiu</i> MARCOV.	21.3			32.3	34.8
<i>Farfugium japonicum</i> (L.) KITAM.	32.0			30.0	31.3
<i>Rohdea japonica</i> (THUNB.) ROTH	37.8			29.7	31.0
<i>Lathyrus japonicus</i> WILLD.	19.1			21.2	27.3
<i>Trachycarpus fortunei</i> (HOOK.) H. WENDL.	19.4			30.8	27.1
<i>Cinnamomum sieboldii</i> MEISN.	16.3			31.2	25.5
<i>Terminalia chebula</i> RETZ.	18.8			29.5	22.2
<i>Ilex latifolia</i> THUNB.	62.9			12.4	18.4
<i>Prunus mume</i> SIEB. et ZUCC.	16.8			12.8	18.3
<i>Buxus microphylla</i> SIEB. et ZUCC. var. <i>japonica</i> (MUELL. ARG.) REHD. et WILS.	24.5			20.0	16.4
<i>Raphiolepis umbellata</i> MAKINO var. <i>mertensii</i> MAKINO	25.2			19.0	11.6
<i>Gardenia jasminoides</i> ELLIS forma <i>grandiflora</i> (LOUR.) MAKINO	30.8			6.9	8.9
<i>Cycas revoluta</i> THUNB.	17.1			8.8	8.8
<i>Prunus tomentosa</i> THUNB.	16.5			<5.0	8.3
<i>Ophiopogon ohwii</i> OKUYAMA	21.2			6.7	5.6
<i>Aspidistra elatior</i> BLUME	26.2			5.6	<5.0
<i>Daphniphyllum teijsmannii</i> ZOLL.	22.9			5.0	<5.0
<i>Olea europaea</i> L.	28.9			<5.0	<5.0
<i>Dendropanax trifidus</i> (THUNB.) MAKINO	23.6			<5.0	<5.0
<i>Torreya nucifera</i> (L.) SIEB. et ZUCC.	25.1			<5.0	<5.0
<i>Laurus nobilis</i> L.	25.7			<5.0	<5.0
<i>Liriope minor</i> (MAXIM.) MAKINO	18.0			<5.0	<5.0
<i>Camellia sasanqua</i> THUNB.	27.0			<5.0	<5.0
<i>Lindera strychnifolia</i> (SIEB. et ZUCC.) F. VILL.	18.4			<5.0	<5.0
<i>Pittosporum tobira</i> (THUNB.) AIT.	21.6			<5.0	<5.0
<i>Glehnia littoralis</i> FR. SCHM., ex. MIQ.	25.6			<5.0	<5.0
<i>Lycoris radiata</i> (L'HERIT.) HERB.	10.0			<5.0	<5.0
<i>Eurya japonica</i> THUNB.	22.2			<5.0	<5.0
<i>Eriobotrya japonica</i> (THUNB.) LINDL.	25.3			<5.0	<5.0
<i>Stauntonia hexaphylla</i> (THUNB.) DECAISNE	28.9			<5.0	<5.0
<i>Daphniphyllum macropodum</i> MIQ.	23.2			<5.0	<5.0

Each value represents the mean of 3 experiments.

solution was measured at 400 nm. The melanin content was expressed as $\mu\text{g}/10^5$ cells.

RESULTS

Anti-tyrosinase Activities of 50% Ethanolic Extracts from Leaves of 38 Plants As shown in Table I, the extracts from the leaves of *P. yedoensis*, *P. zippeliana*, *P. amygdalus*, *P. persica*, *P. armeniaca*, *Thea sinensis* and *Chaenomeles sinensis* among 38 plants collected in the herb garden of Kinki University, exhibited powerful inhibition on tyrosinase activity.

Anti-tyrosinase Activity of Benzaldehyde Benzaldehyde inhibited the enzyme activity and the 50% inhibitory concentration (IC_{50} ; μM) determined by the Litchfield-Wilcoxon's method was 1051.4 μM . The IC_{50} value of the kojic acid, positive control was 19.1 μM .

Effects of Leaves of *Prunus* Plants on the Production of Melanin from Dopachrome by Autoxidation As shown in Table II, the extracts from leaves of *P. yedoensis* and *P. zippeliana* among *Prunus* plants used in this experiment exhibited an inhibitory effect on the production of melanin from dopachrome by autoxidation. The positive control, kojic acid, also inhibited this production.

Effect of Leaves of *P. zippeliana* on the Tyrosinase Activity and Melanin Content of Cultured B-16 Mouse Melanoma Cells As shown in Figs. 1 and 2, the extract from leaves of *P. zippeliana* exhibited an inhibitory effect on tyrosinase activity and the melanin content of cultured B-16 mouse melanoma cells. Kojic acid exhibited a strong inhibitory effect.

TABLE II. Effects of 50% Ethanolic Extracts from *Prunus* Plants and Kojic Acid on the Production of Melanin from Dopachrome by Autoxidation

Sample	Concentration ($\mu\text{g}/\text{ml}$)	Melanin content ($\mu\text{g}/\text{ml}$)	Inhibition (%)
Control	—	82.4 ± 2.7	—
<i>Prunus yedoensis</i> MATSUM.	200	77.8 ± 3.3	5.6
	500	69.8 ± 1.4^a	15.2
	1000	54.3 ± 0.7^b	34.1
<i>Prunus zippeliana</i> MIQ.	200	70.9 ± 2.1	13.9
	500	58.5 ± 1.1^b	29.1
	1000	57.6 ± 0.4^b	30.1
<i>Prunus amygdalus</i> STOKES	200	75.9 ± 0.9	7.9
	500	85.5 ± 1.3	<5.0
	1000	87.8 ± 1.9	<5.0
<i>Prunus armeniaca</i> L. var. <i>ansu</i> MAXIM.	200	93.6 ± 2.4	<5.0
	500	106.6 ± 0.4	<5.0
	1000	106.0 ± 0.4	<5.0
<i>Prunus persica</i> SIEB. et ZUCC.	200	85.8 ± 2.5	<5.0
	500	98.7 ± 1.0	<5.0
	1000	105.4 ± 0.6	<5.0
<i>Prunus mume</i> SIEB. et ZUCC.	200	88.2 ± 5.2	<5.0
	500	98.7 ± 1.2	<5.0
	1000	109.3 ± 1.8	<5.0
Kojic acid	200	77.5 ± 3.1	5.9
	500	59.1 ± 1.4^b	28.2
	1000	35.9 ± 1.1^b	56.4

Tyrosinase was preincubated with dopa at 25°C for 5 min prior to incubation with test substances for 60 min, and the melanin produced was measured. Each value represents the mean $\pm$ S.E. of 3 experiments. Significantly different from the control value, a) $p < 0.05$, b) $p < 0.01$.

Anti-tyrosinase Activities of 50% Ethanolic Extracts from Crude Drugs from the *Prunus* Plant As shown in Table III, the extract from Pruni Cortex and Persicae

Fig. 1. Inhibitory Effects on Tyrosinase Activity in Cultured B-16 Mouse Melanoma of 50% Ethanolic Extracts from the Leaves of *P. zippeliana* and Kojic Acid

Each value represents the mean of 3 experiments.

Fig. 2. Inhibitory Effects on the Melanin Content in Cultured B-16 Mouse Melanoma of 50% Ethanolic Extracts from the Leaves of *P. zippeliana* and Kojic Acid

Each value represents the mean of 3 experiments.

TABLE III. Effects on Tyrosinase Activity of 50% Ethanolic Extracts from Crude Drugs Originated from *Prunus* Plants

Sample	Concentration ($\mu\text{g}/\text{ml}$)	Absorbance ($\times 1000$)	Inhibition (%)
Control	—	1021 ± 16	—
Pruni cortex	50	755 ± 8^b	26.0
	200	392 ± 13^b	61.7
	500	305 ± 7^b	70.1
Persicae semen	50	746 ± 27^b	27.0
	200	605 ± 6^b	40.7
	500	457 ± 6^b	55.3
Armeniaca semen	50	997 ± 4	<5.0
	200	809 ± 7^b	20.8
	500	639 ± 4^b	37.5
Sweet almond	50	928 ± 14^a	9.1
	200	1012 ± 11	<5.0
	500	1007 ± 9	<5.0

Tyrosinase was preincubated with test substances at 25°C for 10 min prior to incubation with dopa for 5 min, and absorbance was read at 475 nm. Each value represents the mean $\pm$ S.E. of 3 experiments. Significantly different from the control value, a) $p < 0.05$, b) $p < 0.01$.

TABLE IV. Effects on the Production of Melanin from Dopachrome by Autoxidation of 50% Ethanol Extracts of Crude Drugs from *Prunus* Plants

Sample	Concentration (μg/ml)	Melanin content (μg/ml)	Inhibition (%)
Control	—	82.4 ± 2.7	—
Pruni Cortex	200	44.6 ± 1.3 ^{b)}	45.8
	500	28.3 ± 3.3 ^{a)}	65.6
	1000	6.5 ± 2.6 ^{b)}	92.0
Persicae Semen	200	72.5 ± 0.8 ^{a)}	12.0
	500	68.9 ± 0.3 ^{b)}	16.4
	1000	64.7 ± 0.5 ^{b)}	21.2
Armeniacae Semen	200	75.5 ± 1.3	8.4
	500	72.3 ± 0.6 ^{a)}	12.2
	1000	72.8 ± 1.0 ^{a)}	11.7
Sweet almond	200	82.5 ± 0.9	<5.0
	500	87.3 ± 2.1	<5.0
	1000	91.2 ± 0.2	<5.0

Tyrosinase was preincubated with dopa at 25°C for 5 min prior to incubation with test substances for 60 min, and the melanin produced was measured. Each value represents the mean ± S.E. of 3 experiments. Significantly different from the control value, a) $p < 0.05$, b) $p < 0.01$.

Semen exhibited a powerful inhibition of tyrosinase activity. The extract of Armeniacae Semen produced a weak inhibition but not sweet almond.

Effects of 50% Ethanol Extracts from Crude Drugs from *Prunus* Plant on the Production of Melanin from Dopachrome by Autoxidation As shown in Table IV, the extract from Pruni Cortex exhibited a powerful inhibition of the production of melanin from dopachrome by autoxidation. The extracts from Persicae Semen and Armeniacae Semen produced weak inhibition but not sweet almond.

DISCUSSION

The purpose of the present investigation was to study the inhibitory effects of leaves from 38 plants and 4 crude drugs on melanin biosynthesis, closely related to hyperpigmentation, in order to develop a skin-whitening agent from natural sources.

Melanin biosynthesis involves the production of dopachrome from dopa by tyrosinase and melanin production from dopachrome by autoxidation. Accordingly, a study was conducted of the inhibitory effect on tyrosinase activity of leaves from 38 plants collected in the herbal garden of Kinki University. Our results showed that, among 38 plants, the 50% ethanolic extract from the leaves of *Prunus* plants, especially *P. yedoensis* and *P. zippeliana*, produced marked inhibition. The leaves of *Thea sinensis* and

Chaenomeles sinensis also produced potent inhibition. *P. yedoensis* and *P. zippeliana*, among the *Prunus* plants used in these experiments, produced inhibitory effects on melanin production by autoxidation. Furthermore, anti-tyrosinase activity and inhibition of melanin production by leaves of *P. zippeliana* were observed in cultured B-16 mouse melanoma cells. There are many reports on the inhibitory effects of crude drugs on tyrosinase activity.⁹⁾ However, as far as crude drugs were concerned, until now only the Bearberry leaf (leaf of *Arctostaphylos uva-ursi*) was known to inhibit melanin production by autoxidation.¹⁰⁾ It is very interesting that the leaves of *P. yedoensis* and *P. zippeliana* produced the inhibition of both tyrosinase activity and melanin production by autoxidation.

Since the leaves of *P. zippeliana* exhibited an inhibitory effect on melanin biosynthesis, the anti-tyrosinase effect of benzaldehyde in *P. zippeliana* was determined. Benzaldehyde produced inhibition of tyrosinase activity and it is likely that benzaldehyde may be one of the active components of *P. zippeliana* producing inhibition of melanin biosynthesis.

In addition, the effects of 4 crude drugs from *Prunus* plants on tyrosinase activity were investigated. It was found that Pruni Cortex and Persicae Semen exhibited a marked inhibitory activity. Further study is in progress to identify the active components of these crude drugs.

The present study clearly demonstrates that *P. zippeliana* possesses the ability to inhibit both tyrosinase activity and melanin production by autoxidation. We suggest that our findings have potentially important implications for the therapeutic use of agents from *Prunus*, especially *P. zippeliana*, in hyperpigmentation.

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