J Wood Sci (2007) 53:153–160
DOI 10.1007/s10086-006-0824-1

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NOTE

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Screening of extracts of Japanese woods for melanin biosynthesis inhibition

Received: January 13, 2006 / Accepted: June 23, 2006 / Published online: October 8, 2006

Abstract The effects of 120 methanol extracts prepared from bark and heartwood of 69 types of Japanese wood on the melanin production of B16 melanoma cells were examined. The melanin content of B16 melanoma cells was determined spectrophotometrically at 405 nm. The extracts were also examined for their effects on cell viability. We found that the methanol extracts of *Fagus crenata* (buna, wood, 100 μg/ml), *Sapium sebiferum* (Nankinhaze, wood, bark, 10 μg/ml), and *Zelkova serrata* (keyaki, wood, 10 μg/ml) greatly inhibited the melanin production of B16 melanoma cells without significant cytotoxicity. However, these extracts did not inhibit tyrosinase activity at the concentration of 100 μg/ml. These findings indicate that the depigmenting mechanism of these extracts involves the suppression of some pigmenting signals in stimulating melanogenesis rather than the inhibition of tyrosinase activity.

Key words Melanin biosynthesis inhibitor · *Fagus crenata* · *Sapium sebiferum* · *Zelkova serrata* · Tyrosinase

Introduction

The products and chemical intermediates derived from natural products are from renewable resources, for example, woody plants. The significance of these compounds, however, does not stop here, for they also have a considerable effect on the utilization of wood. The odor, color, and decay resistance of wood are functions of the extractives. It is worth noting that almost half of all prescription drugs contain substances of natural origin. Even aspirin was first derived in part from willow bark. The investigation of bioactive natural products has, in recent years, assumed a greater sense of urgency in response to the expanding human population and its subsequent demands for food, good health, and increasing areas of land on which to live.

Within the realm of human health, cutaneous hyperpigmentation, including freckles, skin stains, and senile lentigines, is a common pigmentary disorder in humans, which becomes more prominent with aging, especially in Asians. We have focused on the possibility of using natural resources as whitening agents for cosmetic products. An intensive search for a naturally occurring substance that would inhibit melanin pigmentation has been performed.[1] Up to now, most research on the regulation of melanogenesis has focused on the factors affecting tyrosinase, which catalyzes the rate-limiting step of the melanin biosynthesis pathway, specifically, the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and subsequently to DOPA quinone. Kojic acid[2] and arbutin[3] are known as tyrosinase inhibitors and are used as skin-whitening cosmetics. Also, hydroquinone-related compounds have been used as effective depigmentation agents for skin over pigmentation, but they are strong irritants and exhibit cell toxicity.[4]

Recently, it has been reported that some growth factors or cytokines such as basic fibroblast growth factor (bFGF),[5] endothelin-1 (ET-1),[6] and α-melanocyte stimulating hormone (α-MSH)[7] are secreted from ultraviolet (UV)-irradiated keratinocytes or melanocytes and stimulate the proliferation or melanogenesis of human melanocytes. We concluded that we should examine the melanin biosynthesis effect using cultured cells to develop new whitening agents. Also, a mouse melanoma cell line, B16, which produces melanin pigments, has been used extensively for evaluating melanin biosynthesis inhibitors.[8] In the present study, we have examined the inhibitory effect of 120 extracts prepared from 69 Japanese woods on melanin formation and proliferation of B16 melanoma cells.

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Part of this study was presented at the 53rd Annual Meeting of the Japan Wood Research Society, Fukuoka, Japan, March 2003

Materials and methods

Reagents

The chemicals used were L-DOPA, ethylenediaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO), TritonX-100, (Wako, Osaka, Japan); mushroom tyrosinase, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA); teophylline, (Sigma-Aldrich Chemle, Steinhein, Germany); kojic acid, arbutin, (Tokyo Kasei Kogyo, Tokyo, Japan); fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA); Eagle's minimal essential media (EMEM) (Nissui, Tokyo, Japan); and trypsin (Invitrogen, Tokyo, Japan).

Plant materials

Sixty-nine woody species were collected from Kyushu University Forests in Fukuoka and separated into heartwood and bark. All voucher specimens are preserved at the herbarium of the Department of Forest Products, Kyushu University, in Japan. The milled heartwoods or barks (each 10 g) were extracted with methanol (3 × 100 ml) at room temperature for 24 h and the combined extracts were concentrated to dryness. These extracts were dissolved in DMSO for melanin biosynthesis inhibitory tests.

Cell culture

A mouse melanoma cell line, B16, was obtained from the RIKEN Cell Bank. The cell was maintained in EMEM supplemented with 10% (v/v) FBS and 0.09 mg/ml theophyrine. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Inhibitory effect on melanogenesis using cultured B 16 melanoma cell

Confluent cultures of B16 melanoma cells were rinsed in phosphate-buffered saline (PBS) and removed from the plastic using 0.25% trypsin/EDTA. The cells were placed into 24-well plastic culture plates at a density of 1×10^5 cells/well and incubated for 24 h in media prior to being treated with the samples. After 24 h, the medium was replaced with 998 μl of fresh medium and 2 μl of DMSO was added with or without (control) the test sample at various concentrations. The cells were incubated for an additional 48 h, and then the medium was replaced with fresh medium containing each sample. After 24 h, the remaining adherent cells were assayed (see below). Thus, the cells were continuously exposed to the test samples for 3 days.

Determination of melanin content

The melanin content (MC) of cells after treatment was determined as follows. After removing the medium and washing cells with PBS, the cell pellet was dissolved in 1.0 ml of 1 N NaOH. The crude cell extracts were assayed using a micro plate reader (Bio-Tek, USA) at 405 nm to determine melanin content. Results from samples were analyzed as percent of control culture.[9] Kojic acid (500 μg/ml) and arbutin (100 mg/ml) were used as a positive standard.[3]

Cell viability

Cell viability (CV) was determined by use of the microculture tetrazolium technique (MTT). The MTT assay provides a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolic activity in treated cells versus control cells.[3] Culture was initiated in 24-well plates at 1×10^5 cells per well. After incubation, 50 μl of MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide in PBS (5 mg/ml)] was added to each well. The plates were incubated in a humidified atmosphere of 5% of CO_2 at 37°C for 4 h. After removing the medium, formazan crystals were dissolved in 1.0 ml of 0.04 N HCl, and the absorbance was measured at 570 nm relative to 630 nm.

Tyrosinase assay using mushroom tyrosinase

Although mushroom tyrosinase differs somewhat from other sources, this fungal source was used for the present experiment because of its ready availability. It should be noted that the commercial tyrosinase was reported to contain numerous proteins in addition to tyrosinase,[10] but was used without purification. The temperature was controlled at 25°C using an Ecoline E100 circulating bath (Lauda, Germany) with a heater and digital thermometer. The reaction was started by adding the enzyme. Although tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, the assay was carried out in air-saturated solution. Kojic acid was used as a positive control.[11] The sample was first dissolved in DMSO and used for the actual experiment at 30-fold dilution. Controls (without inhibitor) containing DMSO at that concentration were routinely prepared. The assay was performed as previously described.[8] First, 333 μl of 2.5 mM L-DOPA solution was mixed with 600 μl of 0.1 M phosphate buffer ($Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$) (pH 6.5) and incubated at 25°C. Then 33 μl of the aqueous solution of mushroom tyrosinase (1380 U/ml) was added to the mixture, and we immediately measured the initial rate of linear increase in optical density at 475 nm on the basis of dopachrome formation, using aV530 spectrophotometer (Jasco, Japan). The activity was calculated with the following equation:

$$\text{Tyrosinase activity}\,(\%) = \left(\Delta A_{\text{test sample}} / \Delta A_{\text{control}}\right) \times 100$$

Tyrosinase assay using cell extracts from B16 melanoma

B16 melanoma cells were seeded at 2×10^5 cells/ml, in 24-well flat-bottom plate (Falcon) overnight for attachment.

To prepare a solubilized tyrosinase extract for enzyme activity, 398 μl of 50 mM phosphate buffer (pH 6.8) containing 1% Triton X-100 was added and cell pellets were sonicated to solubilize the cell. Afrer 10 min, 20% L-DOPA solution and 2 μl of sample solution were added and cells were incubated at 37°C for 3 h. After this time, the absorbance at 405 nm was measured to determine melanin content.

Results and discussion

We examined the effect of 120 methanol extracts prepared from several parts of 69 woody species collected from the Kyushu University Forests in Fukuoka on melanin biosynthesis (Table 1, Fig. 1). The meal of heartwood and bark of Japanese trees were extracted with methanol at room temperature. Each of the methanol extracts was examined for any melanin biosynthesis inhibitory effect at various concentrations.

After treatment of B16 melanoma cells with various samples for 3 days, cells were harvested and two different parameters of cellular function were measured by microassay, namely the effects on viability and on melanin content. An important concept when selecting bioactive extracts that modulate skin pigmentation is that, for obvious reasons, they should have minimal effects on cell proliferation and/or survival. In these experiments, effects on cell proliferation were assessed using the MTT assay, and effects on melanin content were measured by absorbance at 405 nm (Table 1, Fig. 1). To find a possible candidate for a whitening agent, we established a standard for desirable extracts that they should inhibit melanin content by more than 65% and proliferation by less than 15% at the specific concentration.

To pick out more desirable ingredients, more specifically a safe and effective whitening agent, extracts that showed the percentage of melanin content is 20% or more lower than that of cell viability (e.g. CV – MC ≧ 20, were judged as possible active ingredients, and classified as type A. At first, each of the methanol extracts was examined at a concentration of 100 μg/ml. Extracts were considered as possible candidates based on their effects on B16 melanoma cells (Table 1). These were: *Acer buergerianum* (wood), *Acer palmatum* (wood, bark), *Ilex crenata* (wood, bark), *Ilex pedunculosa* (bark), *Ilex rotunda* (bark), *Dendropanax trifidus* (wood), *Kalopanax septemlobus* (wood), *Viburnum odoratissimum* (wood), *Picrasma quassioides* (wood), *Cornus brachypoda* (wood), *Elaeocarpus japonicus* (bark), *Lyonia ovalifolia* var. *elliptica* (wood), *Aleurites cordana* (wood, bark), *Castnea crenata* (bark), *Fagus crenata* (wood), *Lithocarpus edulis* (wood), *Quercus acutissima* (wood), *Quercus myrsiniefolia* (wood), *Idesia polycarpa* (bark), *Ginkgo biloba* (wood, bark), *Cinnamomum camphora* (bark), *Machilus japonica* (bark), *Machilus thunbergii* (wood), *Ficus arecta* (bark), *Chionanthus retusus* (bark), *Fraxinus griffitbii* var. *japonica* (bark), *Fraxinus mandshurica* (wood), *Ligustrum japonicum* (wood), *Osmanthus heterophyllus* (wood, bark), *Picea jezoensis* var. *bondoensis* (wood), *Picea jezoensis* (wood), *Prunus jamasakura* (bark), *Laurocerrasus zippeliana* (bark), *Photinia serrulata* (wood, bark), *Plotosus lineatus* (wood), *Eurya japonica* (wood), *Stewartia monadelpha* (wood), *Tilia japonica* (wood), *Tilia miqueliana* (bark), and *Zelkova serrata* (bark). As shown in Table 1, most of the extracts failed to match our standard (less than 35% of melanin content, more than 85% of cell viability) for judgment as an active ingredient at the concentration of 100 μg/ml. Only *Fagus crenata* (buna, wood, MC 29%, CV 86%) surpassed our standard.

Some extracts showed neither cell cytotoxicity nor melanin biosynthesis inhibitory activity at the concentration of 100 μg/ml. In those cases, it still was possible that the extracts would have melanin biosynthesis inhibitory activity at a concentration higher than 100 μg/ml. Therefore, those extracts that inhibited the growth of B16 melanoma cells less than 30% and inhibited melanin biosynthesis less than 30%, classified as type B, were reexamined at a concentration of 200 μg/ml. Also, some extracts classified as type A at the concentration of 100 μg/ml were reexamined. It should be noted that some extracts were not reexamined because of limited sample availability. Several extracts seemed to be possible candidates, based on their effects on B16 melanoma cells (Table 1) as follows: *Ilex crenata* (wood), *Ilex pedunculosa* (wood), *Quercus myrsiniefolia* (wood), *Quercus phillyraeoides* (wood), *Chionanthus retusus* (wood, bark), *Fraxinus mandshurica* (wood), *Ligustrum japonicum* (bark), *Osmanthus heterophyllus* (wood), *Laurocerrasus zippeliana* (bark), *Photinia serrulata* (wood, bark), *Photinia glabra* (wood), and *Symplocos mytracea* (kuroki). However, there were no extracts that surpassed our established standard (less than 35% melanin content, more than 85% cell viability) at 200 μg/ml.

We also found that some extracts showed cytotoxicity of more than 30% versus the control at 100 μg/ml, which prohibited our evaluation of their effects on melanin biosynthesis of B16 melanoma cells. Therefore, extracts that inhibited growth of B16 melanoma cells more than 30% versus the

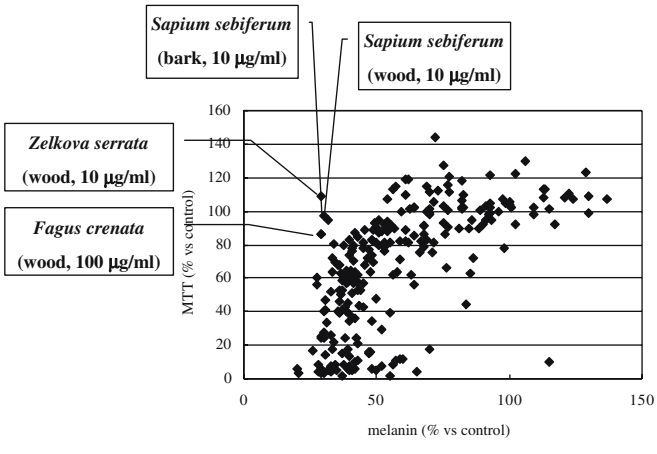

Fig. 1. Effects of woods extracts on melanin content and cell viability of B16 melanoma cells

Table 1. Inhibitory effect of methanol extracts of Japanese woods on melanin biosynthesis and cell proliferation of B16 melanoma cells (final concentration is 10, 50, 100, 200 μg/ml)

Local name	Family	Scientific name	Part used	200 μg/ml			100 μg/ml			50 μg/ml			10 μg/ml		
				MC	CV	Type[a]	MC	CV	Type	MC	CV	Type	MC	CV	Type
Urikaede	Aceraceae	*Acer crataedifolium*	Wood	–	–	–	45	43	C	83	102	B	–	–	–
			Bark	–	–	–	50	5	C	30	27	C	109	98	B
Toukaede	Aceraceae	*Acer buergerianum*	Wood	42	59	C	75	103	A,B	–	–	–	–	–	–
			Bark	49	59	C	93	105	B	–	–	–	–	–	–
Irohamomiji	Aceraceae	*Acer palmatum*	Wood	–	–	–	82	118	A,B	–	–	–	–	–	–
			Bark	–	–	–	41	71	A	–	–	–	–	–	–
Hiiragimochi	Aquifoliaceae	*Ilex cornuta*	Wood	–	–	–	115	10	C	–	–	–	–	–	–
			Bark	–	–	–	20	6	C	27	60	A,C	117	92	B
Mametsuge	Aquifoliaceae	*Ilex crenata* var. *crenata*	Wood	–	–	–	113	108	B	–	–	–	–	–	–
Inutsuge	Aquifoliaceae	*Ilex crenata*	Wood	59	99	A	68	91	A	–	–	–	–	–	–
			Bark	–	–	–	61	110	A	–	–	–	–	–	–
Soyogo	Aquifoliaceae	*Ilex pedunculosa*	Wood	45	78	A	109	102	B	–	–	–	–	–	–
			Bark	–	–	–	61	119	A	–	–	–	–	–	–
Kuroganemochi	Aquifoliaceae	*Ilex rotunda*	Wood	–	–	–	33	5	C	34	18	C	87	72	B
			Bark	–	–	–	53	79	A	–	–	–	–	–	–
Kakuremino	Araliaceae	*Dendropanax trifidus*	Wood	36	52	C	42	87	A	–	–	–	–	–	–
			Bark	–	–	–	43	21	C	55	92	A	–	–	–
Harigiri	Araliaceae	*Kalopanax septemlobus*	Wood	40	8	C	70	101	A,B	–	–	–	–	–	–
Shirakaba	Betulaceae	*Betula platyphylla* var. *japonica*	Wood	–	–	–	92	98	B	–	–	–	–	–	–
Sangojyu	Caprifoliaceae	*Viburnum odoratissimum*	Wood	–	–	–	53	81	A	–	–	–	–	–	–
			Bark	–	–	–	–	–	–	56	89	A	–	–	–
Katsura	Cercidiphyllum	*Cercidiphyllum japonicum*	Bark	–	–	–	40	34	C	37	41	C	103	123	A,B
Nigaki	Cimaroubaceae	*Picrasma quassioides*	Wood	–	–	–	42	64	A,C	67	82	D	–	–	–
			Bark	–	–	–	37	2	C	21	4	C	83	110	A,B
Kanrenboku	Cornaceae	*Camptotbeca acuminata*	Wood	–	–	–	69	83	D	–	–	–	–	–	–
Kumanomizuki	Cornaceae	*Cornus brachypoda*	Wood	–	–	–	64	102	A	–	–	–	–	–	–
			Bark	–	–	–	57	11	C	70	18	C	100	102	B
Hinoki	Cupressaceae	*Chamaecyparis obtusa*	Wood	–	–	–	32	5	C	39	17	C	93	103	B
			Bark	–	–	–	39	6	C	40	13	C	57	115	A
Kobanmochi	Elaeocarpaceae	*Elaeocarpus japonicus*	Wood	–	–	–	38	24	C	43	44	C	113	113	B
			Bark	–	–	–	42	62	A,C	71	75	B	–	–	–
Nejiki	Ericaceae	*Lyonia ovalifolia* var. *elliptica*	Wood	–	–	–	49	82	A	–	–	–	–	–	–
			Bark	–	–	–	42	10	C	–	–	–	–	–	–
Nankinhaze	Euphorbiaceae	*Sapium sebiferum*	Wood	–	–	–	29	25	C	39	65	A,C	30	88	A
			Bark	–	–	–	52	29	C	41	57	C	30	97	A
Aburagiri	Euphorbiaceae	*Aleurites cordana*	Wood	–	–	–	54	107	A	–	–	–	–	–	–
			Bark	–	–	–	50	81	A	–	–	–	–	–	–
Kuri	Fagaceae	*Castnea crenata*	Wood	–	–	–	36	39	C	31	41	C	78	102	A,B
			Bark	–	–	–	45	81	A	–	–	–	–	–	–
Buna	Fagaceae	*Fagus crenata*	Wood	–	–	–	29	86	A	–	–	–	–	–	–
Matebashii	Fagaceae	*Lithocarpus edulis*	Wood	–	–	–	64	102	A	–	–	–	–	–	–

Kunugi	Fagaceae	*Quercus acutissima*	Wood	–	–	–	68	91	A	–	–	–	–	–	–
			Bark	–	–	–	33	26	C	41	76	A	–	–	–
Shirakashi	Fagaceae	*Quercus myrsiniefolia*	Wood	36	68	A,C	70	98	A,B	–	–	–	–	–	–
			Bark	–	–	–	68	79	D	–	–	–	–	–	–
Ubamegashi	Fagaceae	*Quercus phillyraeoides*	Wood	46	89	A	96	100	B	–	–	–	–	–	–
			Bark	–	–	–	56	62	C	77	100	A,B	–	–	–
Konara	Fagaceae	*Quercus serrata*	Wood	34	8	C	93	95	B	–	–	–	–	–	–
			Bark	–	–	–	41	5	C	45	57	C	89	101	B
Abemaki	Fagaceae	*Quercus variabilis*	Wood	–	–	–	26	17	C	72	106	A,B	–	–	–
			Bark	–	–	–	48	34	C	41	51	C	100	104	B
Iigiri	Flacourtiaceae	*Idesia polycarpa*	Bark	–	–	–	63	101	A	–	–	–	–	–	–
Ichou	Ginkgoaceae	*Ginkgo biloba*	Wood	–	–	–	51	80	A	–	–	–	–	–	–
			Bark	–	–	–	53	76	A	–	–	–	–	–	–
Tokiwamansaku	Hamamelidaceae	*Loropetalum chinense*	Wood	–	–	–	47	15	C	86	63	C	98	105	B
			Bark	–	–	–	55	39	C	84	44	C	86	95	B
Kagonoki	Lauraceae	*Actinodaphne laneifolia*	Wood	–	–	–	37	53	C	34	80	A	–	–	–
			Bark	–	–	–	56	8	C	31	34	C	92	121	A,B
Kusunoki	Lauraceae	*Cinnamomum camphora*	Wood	–	–	–	31	47	C	40	64	A	–	–	–
			Bark	–	–	–	62	119	A	–	–	–	–	–	–
Hosobatabu	Lauraceae	*Machilus japonica*	Wood	–	–	–	39	58	C	–	–	–	–	–	–
			Bark	–	–	–	34	72	A	–	–	–	–	–	–
Tabunoki	Lauraceae	*Machilus thunbergii*	Wood	40	39	C	77	116	A,B	–	–	–	–	–	–
			Bark	48	16	C	72	81	B	–	–	–	–	–	–
Niseakashia	Leguminosae	*Robinia pseudo–acacia*	Wood	–	–	–	43	57	C	76	67	C	124	107	B
			Bark	–	–	–	42	6	C	137	107	B	–	–	–
Kobushi	Magnoliaceae	*Magnolia praecocissima*	Wood	–	–	–	81	90	B	–	–	–	–	–	–
			Bark	–	–	–	33	4	C	39	40	C	90	92	B
Hoonoki	Magnoliaceae	*Magnolia bypoleuca*	Wood	–	–	–	64	56	C	130	109	B	–	–	–
			Bark	–	–	–	33	6	C	29	24	C	129	123	B
Inubiwa	Moraceae	*Ficus erecta*	Wood	–	–	–	39	45	C	48	74	A	–	–	–
			Bark	–	–	–	45	68	A,C	64	86	A	–	–	–
Hitotsubatago	Oleaceae	*Chionanthus retusus*	Wood	49	89	A	76	86	B	–	–	–	–	–	–
			Bark	53	93	A	70	99	A,B	–	–	–	–	–	–
Shimatago	Oleaceae	*Fraxinus griffitbii* var. *japonica*	Wood	63	62	C	93	99	B	–	–	–	–	–	–
			Bark	–	–	–	61	82	A	–	–	–	–	–	–
Yachidamo	Oleaceae	*Fraxinus mandshurica*	Wood	40	83	A	75	127	A,B	–	–	–	–	–	–
Nezumimochi	Oleaceae	*Ligustrum japonicum*	Wood	–	–	–	40	74	A	–	–	–	–	–	–
			Bark	51	95	A	85	90	B	–	–	–	–	–	–
Hiiragi	Oleaceae	*Osmanthus heterophyllus*	Wood	36	57	A,C	54	94	A	–	–	–	–	–	–
			Bark	–	–	–	59	82	A	–	–	–	–	–	–
Nataorenoki	Oleaceae	*Osmanthus zentaroanus*	Wood	43	62	C	82	101	B	–	–	–	–	–	–
Touhi	Pinaceae	*Picea jezoensis* var. *bondoensis*	Wood	–	–	–	37	59	A,C	49	93	A	–	–	–
Ezomatsu	Pinaceae	*Picea jezoensis*	Wood	–	–	–	54	88	A	–	–	–	–	–	–
Douglas fir	Pinaceae	*Pseudotsuga menziesii*	Wood	–	–	–	28	8	C	41	79	A	–	–	–
Akamatsu	Pinaceae	*Pinus densiflora*	Wood	–	–	–	30	3	C	59	12	C	122	110	B
			Bark	–	–	–	42	61	C	38	79	A	–	–	–
Yamazakura	Rosaceae	*Prunus jamasakura*	Wood	–	–	–	91	95	B	–	–	–	–	–	–
			Bark	–	–	–	39	60	A,C	34	70	A,C	122	105	B
Bakuchinoki	Rosaceae	*Laurocerrasus zippeliana*	Bark	33	63	A,C	82	106	A,B	–	–	–	–	–	–
Ookanamemochi	Rosaceae	*Photinia serrulata*	Wood	61	89	A	73	112	A,B	–	–	–	–	–	–
			Bark	56	91	A	70	111	A,B	–	–	–	–	–	–

Table 1. *Continued*

Local name	Family	Scientific name	Part used	200 μg/ml			100 μg/ml			50 μg/ml			10 μg/ml		
				MC	CV	Type[a]	MC	CV	Type	MC	CV	Type	MC	CV	Type
Kanamemochi	Rosaceae	*Photinia glabra*	Wood	27	56	A,C	75	93	B	–	–	–	–	–	–
Doronoki	Salicaceae	*Populus maximowiczii*	Wood	–	–	–	30	24	C	37	63	A,C	121	108	B
Mukuroji	Sapindaceae	*Sapindus mukorossi*	Wood	–	–	–	68	86	D	–	–	–	–	–	–
			Bark	–	–	–	29	5	C	35	40	C	114	113	B
Kouyamaki	Sciadopityaceae	*Sciadepitys verticillata*	Wood	–	–	–	29	3	C	33	8	C	96	107	B
Gonzui	Staphyleaceae	*Plotosus lineatus*	Wood	–	–	–	47	77	A	–	–	–	–	–	–
			Bark	–	–	–	42	36	C	40	60	A	102	92	B
Kuroki	Symplocaceae	*Symplocos mytracea*	Wood	38	61	A,C	115	101	B	–	–	–	–	–	–
			Bark	–	–	–	43	11	C	38	8	C	–	–	–
Kouyouzan	Taxodiaceae	*Cunninghamia lanceolata*	Wood	–	–	–	34	22	C	58	64	C	77	90	B
			Bark	–	–	–	60	12	C	36	46	C	91	103	B
Sugi	Taxodiaceae	*Cryptomeria japonica*	Wood	–	–	–	43	53	C	49	115	A	–	–	–
Hisakaki	Theaceae	*Eurya japonica*	Wood	–	–	–	56	113	A	–	–	–	–	–	–
			Bark	–	–	–	31	14	C	–	–	–	–	–	–
Himeshara	Theaceae	*Stewartia monadelpha*	Wood	36	43	C	82	102	A,B	–	–	–	–	–	–
			Bark	–	–	–	55	2	C	42	25	C	106	130	B
Mokkoku	Theaceae	*Ternstroemia gymnanthra*	Wood	–	–	–	65	4	C	52	7	C	84	94	B
			Bark	–	–	–	48	6	C	33	5	C	88	90	B
Sinanoki	Tiliaceae	*Tilia japonica*	Wood	–	–	–	69	100	A	–	–	–	–	–	–
			Bark	–	–	–	35	5	C	37	50	C	130	96	B
Bodaijyu	Tiliaceae	*Tilia miqueliana*	Wood	–	–	–	47	16	C	62	81	D	–	–	–
			Bark	–	–	–	36	68	A,C	66	75	D	–	–	–
Ezoenoki	Ulmaceae	*Celtis jessoensis*	Wood	–	–	–	50	48	C	77	121	A,B	–	–	–
			Bark	–	–	–	40	5	C	29	6	C	130	99	B
Akinire	Ulmaceae	*Ulmus parvifolia*	Wood	–	–	–	30	40	C	51	87	A	–	–	–
			Bark	–	–	–	58	64	C	52	88	A	–	–	–
Keyaki	Ulmaceae	*Zelkova serrata*	Wood	–	–	–	29	4	C	28	4	C	29	105	A
			Bark	–	–	–	41	78	A	–	–	–	–	–	–

–, Not tested; MC, melanin content (%); CV; cell viability (%)

[a] Type A: sample that gave MC 20% (or more) lower than VC (e.g. CV – MC ≧ 20); type B: sample that inhibited the growth of B16 melanoma cells less than 30% and inhibited melanin biosynthesis less than 30%; type C: sample that inhibited the growth of B16 melanoma cells more than 30% versus the control; type D: other

control, classified as type C, were reexamined at a concentration of 50 μg/ml. The possible candidates were: *Ilex cornuta* (bark), *Dendropanax trifidus* (bark), *Viburnum odoratissimum* (bark), *Sapium sebiferum* (wood), *Quercus acutissima* (bark), *Quercus phillyraeoides* (bark), *Quercus variabilis* (wood), *Actinodaphne laneifolia* (wood), *Cinnamomum camphora* (wood), *Ficus erecta* (wood, bark), *Picea jezoensis* var. *bondoensis* (wood), *Pseudotsuga menziesii* (wood), *Pinus denifolia* (bark), *Prunus jamasakura* (bark), *Populus mazimowiczii* (wood), *Plotosus lineatus* (bark), *Cryptomeria japonica* (wood), *Celtis jessoensis* (wood), and *Ulmus parvifolia* (wood, bark). However, there were no extracts that surpassed our established standard (less than 35% of melanin content, more than 85% of cell viability) at 50 μg/ml. However, it is noteworthy that the extract of *Cryptomeria japonica* (wood) inhibited melanin production without cytotoxicity at the concentration of 50 μg/ml because this wood is very common in Japan.

Next, those extracts that inhibited growth of B16 melanoma cells more than 30% versus the control at the concentration of 50 μg/ml were reexamined at a concentration of 10 μg/ml. The extracts that were considered possible candidate are: *Cercidiphyllum japonicum* (bark), *Picrasma quassioides* (bark), *Chamaecyparis obtusa* (bark), *Sapium sebiferum* (wood, bark), *Castnea crenata* (wood), *Actinodaphne laneifolia* (bark), and *Zelkova serrata* (wood). Among them, the extract of *Sapium sebiferum* (wood and bark) and that of *Zelkova serrata* (wood) surpassed our standard at 10 μg/ml (Table 1).

The methanol extracts of *Fagus crenata* (wood, 100 μg/ml), *Sapium sebiferum* (bark and wood, 10 μg/ml), and *Zelkova serrata* (wood, 10 μg/ml) showed the ideal activity, that is, potent melanin biosynthesis inhibitory activity and less cytotoxicity among 120 tested samples (Fig. 1). In spite of the sample crudeness, their activities were stronger than those of kojic acid and arbutin, which are known as potent melanin biosynthesis inhibitors. The inhibitory effect on melanin biosynthesis of kojic acid and arbutin was about 60% at the concentrations of 500 μg/ml and 100 μg/ml, respectively.

To our knowledge, there is little published about the biological activity of the extract of *Fagus crenata*. Chinese tallow tree, *Sapium sebiferum*, is common in Japan as a roadside tree, and its root bark has been used in China as a purgative and diuretic. Several compounds, such as friedelin, sitosterol, and ellagic acid from the leaves, and moretenone, moretenol, and sterols from the stem have been reported.[12] It has been reported that cadalene (7-hydroxy-3-methoxycadalene) extracted from *Zelkova serrata* has high antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilus*, *Streptococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and antioxidant activity and chemopreventive activity against NNK [4-(methylinitrosamino)-1-(3-pyridyl)-1-butanone]–induced lung tumors in A/J mice.[13] In this study, we have found a new facet of biological activity of the extracts of *Fagus crenata, Sapium sebiferum*, and *Zelkova serrata*, that of melanin biosynthesis inhibitory activity (Fig. 1).

Next, we tested the effect of the extracts of *Fagus crenata, Sapium sebiferum*, and *Zelkova serrata* on tyrosinase. Tyrosinase is known to be a key enzyme for melanin biosynthesis in mammalian cells.[14] However, these extracts did not inhibit tyrosinase activity of mushroom tyrosinase or that of the cell-extract of B16 melanoma cells at 100 μg/ml (less than 10% inhibition). In contrast, kojic acid showed a strong inhibitory effect (90% inhibition) on tyrosinase activity at 100 μg/ml. This finding indicated that the depigmenting mechanism of these extracts involves the suppression of some pigmenting signals such as bFGF,[5] ET-1,[6] and α-MSH[7] in stimulating melanogenesis, rather than the inhibition of tyrosinase activity.

To inhibit tyrosinase activity, an inhibitor would have to penetrate both the melanocyte and the melanosomal membranes, because tyrosinase acts within organelles in melanocytes. Therefore, these four extracts [*Fagus crenata* (wood), *Sapium sebiferum* (bark and wood), *Zelkova serrata* (wood)] that did not reduce tyrosinase activity may be more effective as skin-whitening agents than as tyrosinase inhibitors. Recently, several melanin biosynthesis inhibitors without the ability of tyrosinase inhibition have been reported.[15,16] Studies to separate the active compounds contained in these extracts are in progress. The isolation and structural elucidation of active constituents of these extracts will provide not only useful leads in the development of a skin-whitening agent but also ideas about other utilizations of woody plants.

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