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# Melanin Hyperpigmentation Inhibitors from Natural Resources

Hideaki Matsuda, Kazuya Murata, Kimihisa Itoh,
Megumi Masuda and Shunsuke Naruto
*Faculty of Pharmacy, Kinki University*
*Japan*

## 1. Introduction

In Oriental countries, such as China, Korea and Japan, a female beauty criterion since ancient times has been a face with fair skin, and the admiration of women with young, healthy, bright and fair skin has created a whitening cosmetics market. The color of human skin and hair is determined by a number of factors. Biosynthesis of the melanin pigment, namely melanogenesis, is the most important factor. Melanogenesis is a multistage process involving melanin synthesis, melanin transport, and melanosome release. Tyrosinase is one of the key enzymes in the melanin biosynthetic pathway. Abnormal deposition of the melanin pigment causes hyperpigmentary disorders.

From natural sources, a number of ingredients with an inhibitory effect on melanin hyperpigmentation have been found, and some of them were developed as cosmetic agents and over the counter (OTC) drugs in Oriental countries. On the other hand, some medicinal chemists have recently paid a lot of attention to inhibitors of melanin production to prevent hyperpigmentary disorders such as melasma, freckles and age spots. To develop novel and useful cosmetic agents, supplements, functional foods and OTC drugs, we have continued to research regulators of melanin production from natural sources since 1980. We describe here our screening strategy and studies on targeted melanin hyperpigmentation inhibitors from natural plant sources, *e.g.* Umbelliferae, Ericaceae, Rubiaceae, Piperaceae and Rutaceae plants. Interesting findings originating from the screening results are also described.

## 2. The search for cosmetic whitening agents from Chinese herbal medicine

### 2.1 Literature search for cosmetic whitening agents in ancient Chinese herbal books

Recently, a retrospective search for cosmetic agents from traditional crude drugs including plants, animals, and inorganic compounds has become a global trend. Traditional application of herbs and biological components of animals to cosmetics and OTC drugs is based on long experience under the expectation that they exert the attributed physiological action. The historical application of Chinese crude drugs to cosmetics has been described in detail in many ancient Chinese herbal books and literature. Since some herbs used for cosmetics, such as flowers of *Carthamus tinctorius* (safflower) and the juice of *Aloe ferox* (aloe), have been used as crude drugs for thousands of years all over the world, knowledge and experience of these herbs have been accumulated.

The first strategy involves a literature search of ancient Chinese books of traditional Chinese herbal medicine to select target herbs which may have the desired biological activity. Herbs having an injury care effect, blood circulation improvement effect and anti-microbial activity could be applied to cosmetic agents with the expectation that they could exert the attributed activity. It was found that about 200 formulas for cosmetics which could be used to beautify a woman's face were listed in several ancient Chinese books of traditional Chinese herbal medicine about 1,500 years ago. A number of dosage delivery forms, such as creams, lotions, pastes, cologne water, suspensions, and emulations, as well some used in modern cosmetics, for external use are found in these 200 formulas. Several Chinese crude drugs, such as seeds of *Euphorbia lathyris* (caper spurge), flowers of *Prunus salicina* (Chinese plum), seeds of *Adenanthera pavonina* (red sandalwood tree), seeds of *Gleditsia japonica* (honey locust), rhizomes of *Kaempferia galangal* (galangale), and seeds of *Cuscuta japonica* were used as medicines for external use or cosmetics to prevent stains and freckles accompanied by hyperpigmentation. As a result of our literature search, Chinese herbs originating from Umbelliferae plants, *e.g.* roots of *Angelica dahurica* (angelica dahurica root), rhizomes of *Cnidium officinalis* (cnidium rhizome), roots of *Angelica acutiloba* (Japanese angelica root), roots and rhizomes of *Saposhnikovia divaricate* (saposhnikovia root), were most frequently listed for the prevention of hyperpigmentation in the 200 formulas for cosmetics described above. An example of the description of Umbelliferae plants which were used to beautify a woman's face in an ancient Chinese herbal book written in Chinese characters is illustrated in Fig. 1.

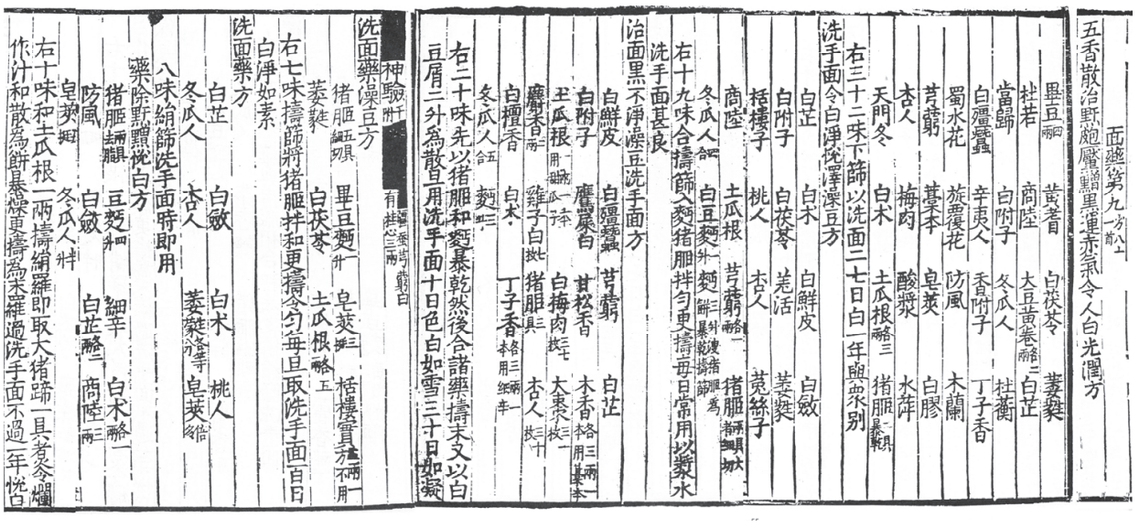


Fig. 1. Description of Umbelliferae plants used to beautify a woman's face in an ancient Chinese herbal book

### 2.2 Tyrosinase inhibitory activity of Umbelliferae plants

Tyrosinase catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-DOPA), followed by the oxidation of L-DOPA to dopaquinone, and oxidative polymerization of several dopaquinone derivatives produces melanin. Thus, the tyrosinase inhibitor is one of candidates for reducing melanogenesis.

In order to find novel ingredients for whitening cosmetics from natural resources, the effect of test samples on melanogenesis was assayed by using mushroom tyrosinase and/or cultured murine B16 melanoma cells (B16 melanoma cells) as the first screening (Mason & Peterson, 1965). On the basis of the first screening results of the selected herbs followed by pharmacological tests, we further examined whether the herbs and their active constituents could be used as cosmetic agents.

From 200 formulas for cosmetics which could be used to beautify a woman's face in the ancient Chinese books of the traditional Chinese herbal medicine, 22 crude drugs were selected as they were most frequently listed in the cosmetics formulas. We examined the tyrosinase inhibitory activity of 50% methanolic extract of the selected 22 crude drugs. Among them, 11 crude drugs originating from Umbelliferae plants exhibited relatively potent tyrosinase inhibitory activity compared to other crude drugs as shown in Table 1 (Masamoto et al., 1980). Although the inhibitory activity of the cited plants was not superior to that of the plants whose potent tyrosinase inhibitory activities have been reported, Umbelliferae plants are generally aromatic, and some of them improve blood circulation and have anti-inflammatory activity. Because of these characteristic physical and pharmacological properties, Umbelliferae plants seemed to be favorable for cosmetics, and were frequently used in the ancient cosmetics formula described in the ancient Chinese herbal books.

| Plant name | Parts | $IC_{50}$ (mg/ml)* |
|---|---|---|
| *Glebnia littoralis* | root | 1.2 |
| *Angelica acutiloba* var. *sugiyamae* | root | 1.8 |
| *Notopterygium forbesii* | rhizome | 3.0 |
| *Peucedanum praeruptorum* | root | 4.4 |
| *Angelica pubescens* | root | 5.1 |
| *Bupleurum falcatum* | root | 5.5 |
| *Ledebouriella seseloides* | root | 6.3 |
| *Ligusticum wallichii* | rhizome | 6.3 |
| *Ligusticum sinense* | rhizome and root | 7.0 |
| *Angelica acutiloba* | root | 7.0 |
| *Cnidium officinale* | rhizome | 9.6 |

Table 1. $IC_{50}$ values of tyrosinase inhibitory activities of 50% methanolic extract obtained from crude Umbelliferae drugs (*; $IC_{50}$ values are indicated as relevant to the concentration of dried crude drugs. *Ref.*; Masamoto et al., 1980)

## 3. The search for cosmetic whitening agents from Alpine plants

### 3.1 Tyrosinase inhibitory activity of Ericaceae plants

Considering the growing environment and characteristic constituents of plants provides another approach to look for novel tyrosinase inhibitors. The second strategy is based on the following consideration. Plants growing in high mountain regions or at the coast of islands in the South Pacific receive stronger solar ultraviolet (UV) radiation all year round than plants growing in other regions, and thus the former plants may have a specific biological self defense system against harmful UV radiation, such as anti-oxidative systems and biosynthesis of several pigments. It has been reported that superoxide dismutase (SOD) is one of the key factors that reduce melanin production caused by UV radiation. These facts

indicate that the anti-oxidative system may play an important role in the regulation of melanogenesis in humans, and that tyrosinase inhibitors with SOD-like activity and/or anti-oxidant activity may be useful ingredients in the field of whitening cosmetics (Tobin & Thody, 1994). Thus, anti-oxidative activity, *e.g.* the SOD-like activity, of some samples was assayed by various methods.

Ericaceae plants grow in arid zones of sub-high mountain regions rich in solar UV radiation. Bearberry (*Arctostaphylos uva-ursi*) was selected from the Ericaceae plants as a screening target because its leaves have historically been used as an anti-septic for the urinary tract in Europe and Japan. A 50% methanolic extract of bearberry leaves showed dose-dependent tyrosinase inhibitory activity. Activity-guided fractionation of the extract led to isolation of arbutin as an active component (Matsuda et al., 1992a). Arbutin (Fig. 2), a major constituent of bearberry, showed a weak tyrosinase inhibitory activity. The activity of the bearberry leaf extract was not fully explained by arbutin, so the extract may have contained other unidentified active components.

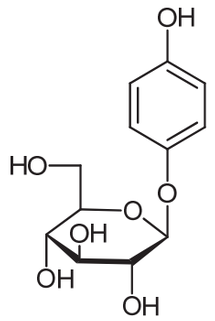



Fig. 2. Arbutin

In order to find a more potent tyrosinase inhibitor than bearberry leaf, the activity of 50% ethanolic leaf extracts obtained from five plants of the genus of *Arctostaphylos e.g. A. patula* (greenleaf manzanita), *A. viscida* (whiteleaf manzanita), *A. canescens* (hoary manzanita), *A. columbiana* (hairy manzanita), and *A. nevadensis* (pinemat manzanita), growing in a sub-high mountain region of North America was assayed (Matsuda et al., 1996). Simultaneously, the SOD-like activity of the five extracts was tested. All exhibited similar SOD-like activity, and the tyrosinase inhibitory activities of the extracts of *A. patula* and *A. viscida* were slightly more potent than that of *A. uva-ursi*, as shown in Table 2. These results indicated that the leaves of *A. uva-ursi*, *A. patula* and *A. viscida* may be useful agents for whitening cosmetics, and it is expected that further screening of Alpine plants may lead to more potent cosmetic whitening agents.

| Plants | Tyrosinase inhibitory activity ($IC_{50}$, μg/ml) | SOD-like activity ($IC_{50}$, μg/ml) |
|---|---|---|
| *A. patula* | 133 | 15.8 |
| *A. viscida* | 145 | 16.0 |
| *A. nevadensis* | 243 | 10.8 |
| *A. columbiana* | 246 | 11.5 |
| *A. canescens* | 226 | 15.3 |
| *A. uva-ursi* | 191 | 19.4 |

Table 2. $IC_{50}$ values of tyrosinase inhibitory and SOD-like activities of 50% methanolic extract from various Arctostaphylos plants (*Ref.*; Matsuda et al., 1996)

### 3.2 The application of cosmetic agents to OTC drugs

Adrenocortical steroids are externally used in the treatment of allergic and atopic dermatitis. These steroid drugs show excellent efficacy, but the long term external use of steroid drugs cause several adverse effects such as skin pigmentation. Although the pigmentation mechanism affected by steroids has not been fully elucidated, some agents with tyrosinase inhibitory and/or anti-oxidant activity may be useful in the prevention of pigmentation.

Bearberry leaf exhibited both tyrosinase inhibitory and anti-oxidative activities as described above. A Japanese lady who visited a Chinese medicinal pharmacy in Osaka told us that her allergic skin eruption caused by hair dye was improved by washing her face with an aqueous extract of bearberry leaf. This information prompted us to examine anti-allergic and anti-inflammatory activities of aqueous extract of bearberry leaf. We investigated the effect of external application of an ointment containing 1 and 2% of bearberry leaf extract to allergic and inflammatory model rodents in comparison with an ointment of 0.005% and 0.025% of dexamethazone, a steroid with potent anti-inflammatory activity (Matsuda et al., 1992b). Bearberry leaf extract did not show anti-allergic and anti-inflammatory activities. However, in the case of external application of a combination ointment of the bearberry extract and dexamethazone, the bearberry extract enhanced the anti-allergic and anti-inflammatory activities of dexamethazone via a synergistic effect without enhancement of adverse effects caused by the steroid. Based on these pharmacological results, Berrybear® ointment was launched on the Japanese OTC drug market after clinical trials by Daiichi Seiyaku Co. Ltd. (present affiliation: Daiichi Sankyo Co. Ltd.) in 2005.

## 4. The search for cosmetic whitening agents from plants in the South Pacific

It was thought that the plants growing at the coast of islands in the South Pacific may have a specific self-defense system against solar UV radiation. Therefore, we looked for cosmetic whitening agents from such plants. We collected a number of plants including tropical and folk medicinal plants at Palau island (The Republic of Palau), Fiji islands (The Republic of Fiji Islands), Tongatapu island (The Kingdom of Tonga), 'Eua island (The Kingdom of Tonga), and Tahiti (French Polynesia) under the approval of the respective governments. Tyrosinase inhibitory activity of the extracts obtained from the collected 150 plants was assayed. Among them, noni (*Morinda citrifolia*, Rubiaceae) was selected for further investigation to find novel cosmetic whitening agents. We simultaneously examined the effect of these extracts on melanogenesis in B16 melanoma cells. In the screening, the extract of kava (rhizome of *Piper methysticum*, Piperaceae) was found to stimulate melanogenesis in B16 melanoma cells, as described in the section 4.2.

### 4.1 *Morinda citrifolia* (Noni)

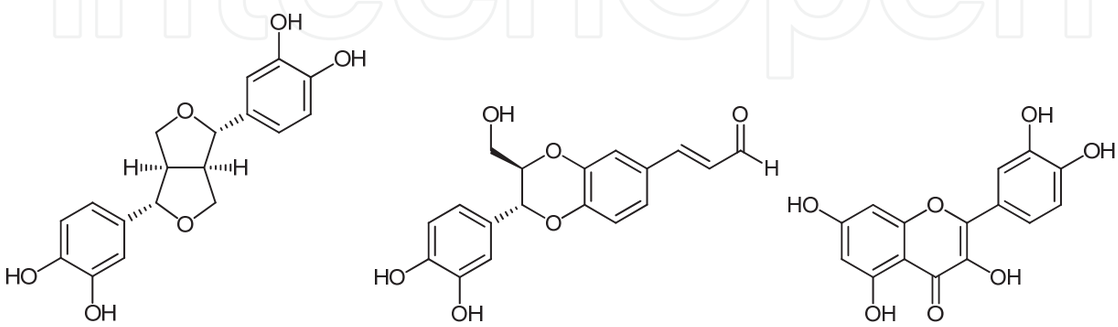
The fruit, roots, bark and leaves of a tropical tree, *Morinda citrifolia*, commonly known as "noni" in Hawaii and Tahiti, have long been used throughout Polynesia as a folk medicine in the treatment of many diseases, *e.g.* hypertension and diabetes. Recently, the juice of the noni fruit and tea made from noni leaves have been launched on the functional food market. Since noni fruit contains a lot of seeds in its flesh, these seeds are removed and discarded during the production process of noni fruit juice. Consequently, we focused on the utility value of noni seeds.

We examined the tyrosinase inhibitory activity and anti-oxidant activity of 50% ethanolic extracts of the fruit flesh, leaves, and seeds of noni, respectively (Masuda et al., 2009). As for anti-oxidant activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of

the extracts were tested. A 50% ethanolic extract from noni seeds (MCS-ext) showed more potent inhibition of tyrosinase and DPPH radical scavenging activities than extracts of noni leaves or flesh. Activity-guided fractionation followed by chromatography of MCS-ext led to the isolation of 3,3′-bisdemethylpinoresinol, americanin A, and quercetin as active constituents with both tyrosinase inhibitory and radical scavenging activities. Americanin A and quercetin also showed SOD-like activity (Table 3 and Fig. 3). In addition, MCS-ext exhibited potent *in vitro* inhibition of elastase, and ursolic acid was a major active constituent of MCS-ext. UV irradiation promotes photoaging of human skin. Chronic UV exposure denatures collagen and elastic fibers in the dermis and induces wrinkles in human skin. In the process of photoaging of human skin, neutrophils play an important role. They infiltrate the skin and release active enzymes such as human leukocyte elastase (HLE), which cleaves the helix structure of type I collagen and then degrades elastic fibers in human skin. Therefore, HLE inhibitors may be useful ingredients for prevention of skin wrinkles. MCS-ext was found to contain, tyrosinase inhibitory, anti-oxidant, and HLE inhibitory active constituents, namely 3,3′-bisdemethylpinoresinol, americanin A, quercetin and ursolic acid. These findings suggested that noni seeds could be a useful ingredient in cosmetics for whitening and/or wrinkle-prevention.

| Samples | Tyrosinase inhibitory activity (mM) | SOD-like activity (μM or U/ml) | Radical-scavenging activity (μM) |
|---|---|---|---|
| Americanin A | 2.7 | 170 μM | 11 |
| 3,3′-Bisdemethylpinoresinol | 0.3 | N.E. | 4 |
| Quercetin | 0.1 | 30 μM | 6 |
| Kojic acid | 0.03 | N.D. | N.D. |
| Arbutin | 83.3 | N.D. | N.D. |
| Superoxide dismutase (SOD) | N.D. | 0.3 U/ml | N.D. |
| L-Ascorbic acid | N.D. | N.D. | 23 |

Table 3. $IC_{50}$ values of tyrosinase inhibitory, SOD-like, and radical-scavenging activities of americanin A, 3,3′-bisdemethylpinoresinol, quercetin, kojic acid, arbutin, superoxide dismutase and L-ascorbic acid (N.D.; Not determined. N.E.; No effect. *Ref.*; Masuda et al., 2009)




3,3′-bisdemethylpinoresinol americanin A quercetin

Fig. 3. 3,3′-Bisdemethylpinoresinol, americanin A and quercetin

### 4.2 Piperaceae plants

During the course of the first screening for the effects of plants collected from islands in the South Pacific on melanogenesis by using B16 melanoma cells, it was found that the extract of rhizome of *Piper methysticum* (Piperaceae) stimulated melanogenesis (Matsuda et al., 2006). The rhizomes are known as kava (kava-kava or kawa) in Oceania, and the South Pacific islanders have traditionally used the rhizome to prepare a psychoactive beverage for social and ceremonial events.

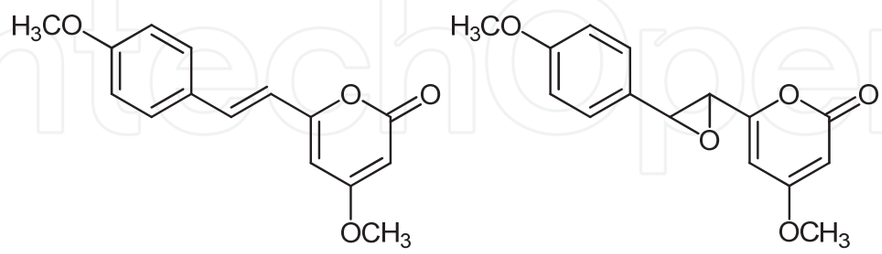
With the increase in the elderly population, many Asian people are develop gray hair. Thus, the cosmetic market for hair-dye and anti-gray hair agents is growing. Hair turning gray is caused by genetic predisposition, aging, reduced melanocytes caused by environmental stress, and reduced biosynthesis of melanin pigment, or melanogenesis. Hair-dye agents are used to treat gray hair, and many anti-gray hair agents are under development. However, there remain some problems with these agents, such as insufficient activity and side effects due to the dyes. Although anti-gray hair agents aim for the opposite effect of cosmetic whitening agents, there is a need for safer anti-gray hair agents that exhibit satisfactory melanogenesis activity and gray hair prevention. Therefore, we continued screening to look for ingredients with more potent melanogenesis stimulation activity among Piperaceae plants.

| Samples | Parts | Concentration (μg/ml) | Melanin content (μg/well) | Cell proliferation (%) |
|---|---|---|---|---|
| Control | | | 11.0 ± 0.8 | 100.0 ± 0.9 |
| *P. methysticum* | Leaf | 1 | 9.3 ± 0.1 | 101.8 ± 0.9 |
| | | 10 | 9.5 ± 0.5 | 106.9 ± 1.4* |
| | Stem | 1 | 10.7 ± 0.4 | 101.7 ± 2.0 |
| | | 10 | 12.8 ± 0.6* | 102.2 ± 1.3 |
| | Rhizome | 1 | 10.6 ± 0.8 | 102.4 ± 2.9 |
| | | 10 | 14.0 ± 0.6* | 108.6 ± 2.0* |
| *P. nigrum* | Leaf | 1 | 13.0 ± 0.3* | 99.9 ± 3.4 |
| | | 10 | 14.9 ± 0.2** | 104.0 ± 2.9 |
| | Stem | 1 | 11.7 ± 0.4 | 98.8 ± 1.0 |
| | | 10 | 11.8 ± 0.4 | 95.9 ± 1.5 |
| | Fruit | 1 | 11.2 ± 0.5 | 99.5 ± 0.6 |
| | | 10 | 15.5 ± 1.1** | 101.6 ± 0.4 |
| Theophylline | | 1 | 12.4 ± 0.3 | 99.1 ± 0.8 |
| | | 10 | 18.7 ± 0.5** | 96.9 ± 1.2 |

Table 4. Effects of 50% ethanolic extracts from Piperaceae plants and theophylline on melanin content in B16 melanoma cells (Each value represents the mean ± S.E. of triplicates. Statistical analysis was performed with a multiple comparison test using the Bonferroni/Dunn algorithm. Significantly different from the control group at *: $p<0.05$, **: $p<0.01$. *Ref.*; Matsuda et al., 2006)

Melanogenesis stimulation activity of 50% ethanolic extracts obtained from several different parts of six Piper species, namely *P. longum* (long pepper), *P. kadsura* (Japanese pepper), *P. methysticum*, *P. nigrum* (black pepper), *P. betle* (betel leaf), and *P. cubeba* (cubeb), were examined (Table 4). Among them, the extracts of kava and *P. nigrum* leaf showed a potent

stimulatory effect on melanogenesis without any significant effect on cell proliferation, as shown in Table 4. Activity-guided fractionation of kava extract by using B16 melanoma cells led to the isolation of two active kavalactones, yangonin and (+)-7,8-epoxyyangonin (Fig. 4 and Table 5). (+)-7,8-Epoxyyangonin showed a significant stimulatory effect on melanogenesis in B16 melanoma cells. Yangonin exhibited a weak melanogenesis stimulation activity.




yangonin (+)-7,8-epoxyyangonin

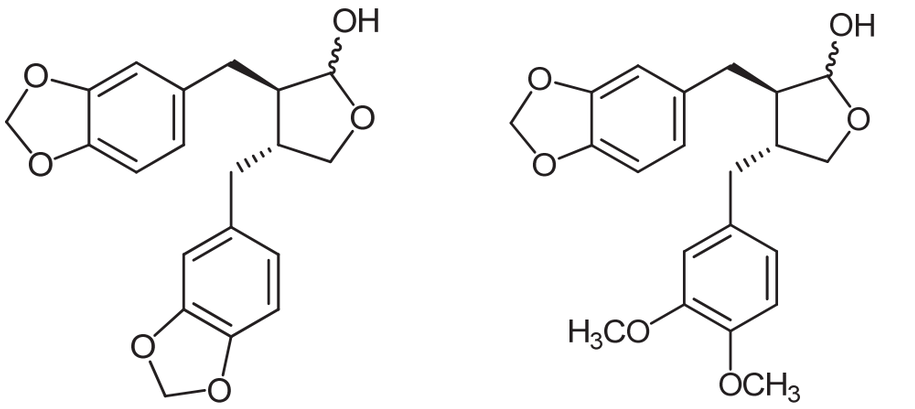
Fig. 4. Yangonin and (+)-7,8-epoxyyangonin

| Samples | Concentration (μg/ml) | Melanin content (μg/well) | Cell proliferation (%) |
|---|---|---|---|
| Control | | 10.6 ± 0.5 | 100.0 ± 1.6 |
| Yangonin | 1 | 13.4 ± 1.2** | 100.6 ± 2.3 |
| | 10 | 17.3 ± 0.5** | 108.2 ± 1.2* |
| (+)-7,8-Epoxyyangonin | 1 | 16.3 ± 1.1** | 107.9 ± 0.2** |
| | 10 | 25.4 ± 0.7** | 116.9 ± 2.8** |
| Theophylline | 1 | 10.2 ± 0.3 | 95.6 ± 1.2 |
| | 10 | 21.0 ± 0.2** | 94.6 ± 2.8 |

Table 5. Effects of yangonin, (+)-7,8-epoxyyangonin and theophylline on melanin content in B16 melanoma cells (Each value represents the mean ± S.E. of triplicates. Statistical analysis was performed with a multiple comparison test using the Bonferroni/Dunn algorithm. Significantly different from the control group at *: $p<0.05$, **: $p<0.01$. *Ref.*; Matsuda et al., 2006)

The *P. nigrum* leaf extract showed the most potent stimulation activity. Fruits of *P. nigrum* are widely used as a pungent spice. The use of Piper leaf has not yet been fully developed. Activity-guided fractionation followed by chromatography of the methanolic leaf extract led to the isolation of two active lignans, (-)-cubebin and (-)-3,4-dimethoxy-3,4-desmethylenedioxycubebin (Fig. 5) (Matsuda et al., 2004). Two lignans showed a significant melanogenesis stimulatory activity without any significant effects on cell proliferation, as shown in Table 6. Therefore, melanogenesis stimulation activity of the leaf extract was attributable to these two lignans. Especially, (-)-cubebin showed the most potent melanogenesis stimulation activity in B16 melanoma cells without any significant effects on cell proliferation. Since (-)-cubebin is a new melanogenesis stimulating substance, we tried to elucidate its melanogenesis stimulation mechanism by using B16 melanoma cells (Hirata et al., 2007). Tyrosinase activity was increased at 24 to 72 h after addition of (-)-cubebin to B16 melanoma cells, and then the intracellular melanin amount

was increased at 48 to 96 h after the treatment. The expression levels of tyrosinase were time-dependently enhanced after the treatment with (-)-cubebin. The activation of microphthalmia-associated transcription factor (MITF), a transcription factor that regulates tyrosinase gene expression, is known to be a critical event during melanogenesis. (-)-Cubebin elevated the level of phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) of which the cascade activates MITF, whereas no effect was observed in the levels of phosphorylation of ERK 1/2 and p70 S6K1. SB203580, a selective inhibitor of p38 MAPK, completely blocked (-)-cubebin-induced expression of tyrosinase mRNA in B16 melanoma cells. These results suggest that one of the mechanisms for (-)-cubebin-induced melanogenesis in B16 melanoma cells is attributable to the increase in tyrosinase gene expression through a positive regulator, MITF, initiated by (-)-cubebin-induced activation of p38 MAPK, as shown in Fig. 6.




(-)-cubebin (-)-3,4-dimethoxy-3,4-desmethylenedioxycubebin

Fig. 5. (-)-Cubebin and (-)-3,4-dimethoxy-3,4- desmethylenedioxycubebin

| Samples | Concentration (μM) | Melanin content (μg/well) | Cell proliferation (%) |
|---|---|---|---|
| Control | | 7.1 ± 0.3 | 100.0 ± 2.6 |
| (-)-Cubebin | 0.3 | 8.6 ± 0.9 | 101.1 ± 2.5 |
| | 1.0 | 10.7 ± 0.2** | 102.5 ± 2.1 |
| | 3.0 | 11.8 ± 0.1** | 101.1 ± 0.9 |
| (-)-3,4-Dimethoxy-3,4-des-methylenedioxycubebin | 0.3 | 9.1 ± 0.3 | 98.9 ± 0.9 |
| | 1.0 | 10.7 ± 0.2* | 103.6 ± 1.4 |
| | 3.0 | 8.8 ± 0.3 | 109.9 ± 2.0* |
| Theophylline | 3.0 | 9.3 ± 1.0* | 105.5 ± 6.1 |
| | 10.0 | 8.3 ± 0.1 | 99.3 ± 1.1 |

Table 6. Effects of (-)-cubebin, (-)-3,4-dimethoxy-3,4-desmethylenedioxycubebin and theophylline on melanin content in B16 melanoma cells (Each value represents the mean ± S.E. of triplicates. Statistical analysis was performed with a multiple comparison test using the Bonferroni/Dunn algorithm. Significantly different from the control group at *: $p<0.05$, **: $p<0.01$. *Ref.*; Matsuda et al., 2004)

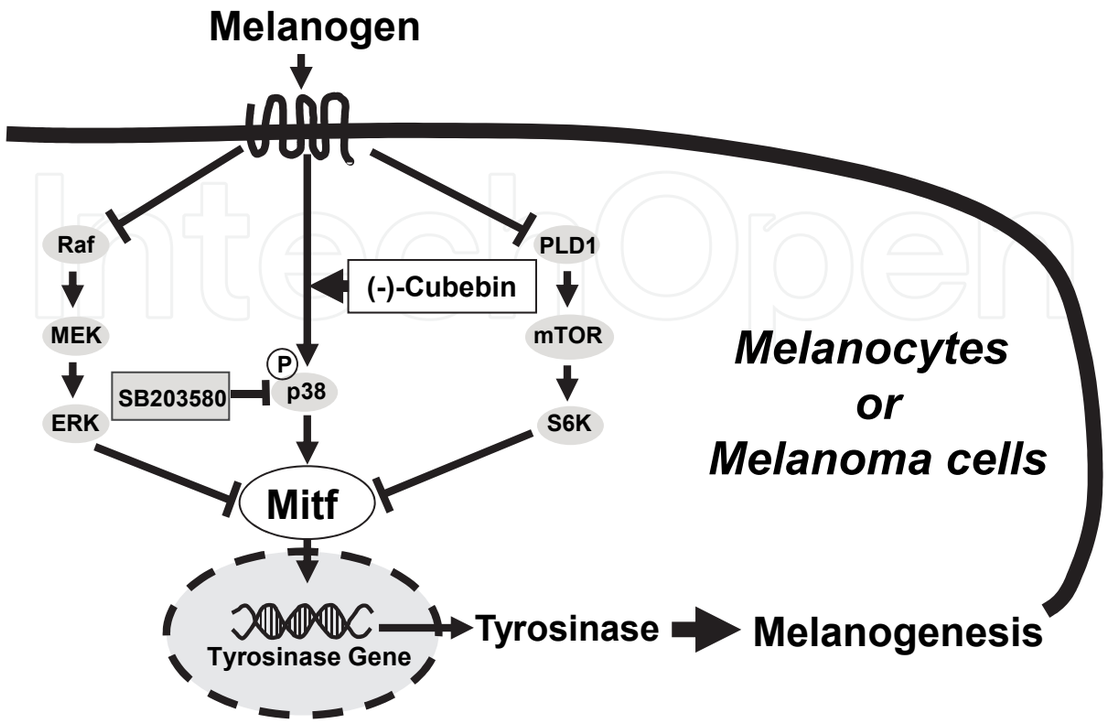



Fig. 6. Proposed scheme showing the activation mechanism of (-)-cubebin on the melanogenesis signaling pathway

Melanogenesis stimulating agents were found during the course of the screening using B16 melanoma cells for cosmetic ingredients with melanogenesis inhibitory activity. Some of the stimulation agents that originated in Piper plants may be useful as cosmetic ingredients for prevention of gray hair after further experimental studies, including clinical trials.

## 5. The search for cosmetic whitening agents from Citrus fruits

### 5.1 Citrus fruits are used as crude drugs throughout the world

Fruits of *Citrus*, *Fortunella*, and *Poncirus* genera (Rutaceae) are generally called Citrus fruits. Yellowish ripe fruits belonging to *Citrus* and *Fortunella* genera are popular, juicy and sour foods that are eaten all over the world. As for the historical medicinal use of Citrus fruits, the ancient Egyptians used them as an anti-bacterial mummification agent. The fruits were also used as insecticides and antidotes, and for the treatment of frostbite, external wounds and colds in the ancient Rome. It has been reported that the fruits were used as washing agents for hair and copper goods in the ancient India. Citrus fruits have been used as crude drugs from ancient times in China as well as other regions; for example, an illustration of Citrus-like fruit (Fig. 7) has been depicted in the oldest Chinese herbal book. In later years, unripe fruits of *C. aurantium* (bitter orange) and *C. natsudaidai* (natsudaidai), and the peals of the ripe fruits of *C. aurantium*, *C. natsudaidai*, and *C. unshiu* (satsuma mandarin) were described in several Chinese herbal books. All have historically been used as digestive medicines, cough remedies, and expectorants.

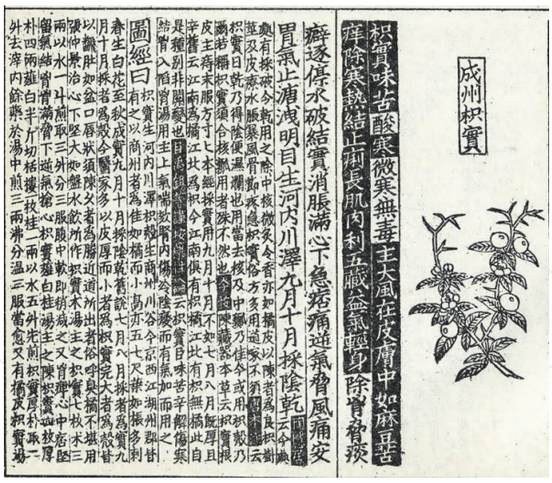



Fig. 7. Description on Citrus fruits in an ancient Chinese herbal book

### 5.2 Citrus plants

The anatomical taxonomy of Citrus plants is controversial because cultivated variations of Citrus plants for new entry into the fruit market increase year by year with recent improvements in breeding. On the other hand, chemotaxomomical classification of Citrus fruits based on our high performance liquid chromatography (HPLC) analysis (Kubo et al., 2004) of four flavanone glycosides (Fig. 8) in the fruits revealed that Citrus fruits could be classified into the following four groups; 1) a group in which the major flavanone glycosides are narirutin and hesperidin with a rutinoside moiety: 2) a group in which the major glycosides are naringin and neohesperidin with a neohesperidoside moiety: 3) a group in which the major glycoside is naringin: 4) a group in which none of the flavanone glycosides described above were detected. We reported that the content of the four flavanone glycosides cited above in unripe fruits was higher than that in ripe ones (Kubo et al., 2004), while the flavanone glycosides content in peel was higher than that in edible flesh.

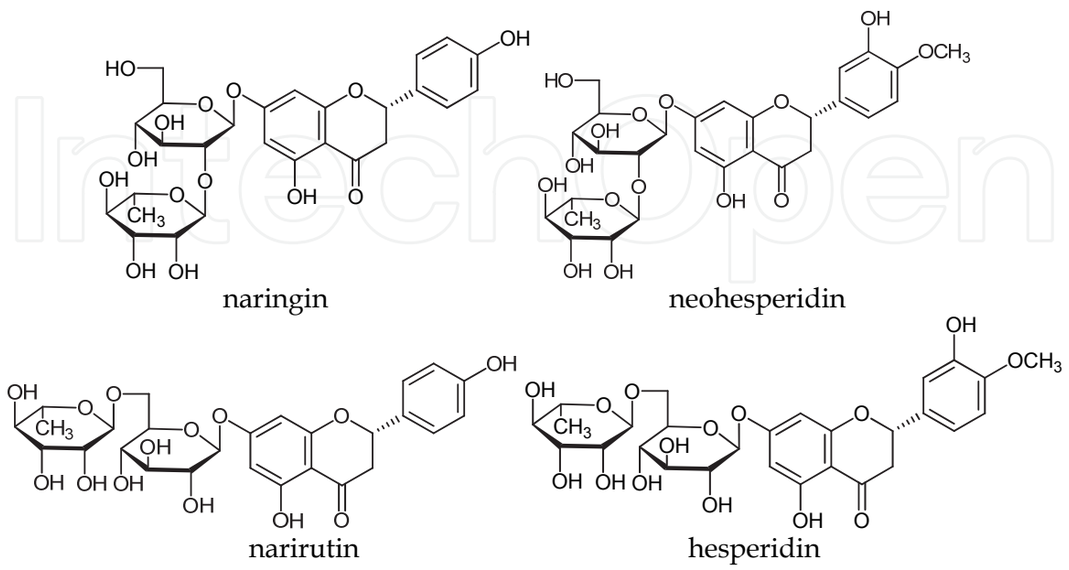



Fig. 8. Flavanone glycosides of Citrus fruits

Flavonoid compounds exhibit various biological activities, such as vasodilatory, anti-oxidative and radical scavenging activities. Since the peel of Citrus fruits is exposed to solar UV radiation, it was assumed that flavanone glycosides may exert an anti-photoaging effect by preventing sun damage. This assumption prompted us to examine the tyrosinase inhibitory activity of some Citrus fruits.

From the most popular Citrus fruits, *C. unshiu* fruit and *C. hassaku* (hassaku) fruit were selected as representatives of the group in which the major flavanone glycosides are narirutin and hesperidin and the group in which major glycosides are naringin and neohesperidin, respectively. Assay results for each 50% ethanolic extract, including seasonal variations in activity, are shown in Table 7 (Itoh et al., 2009). Tyrosinase inhibitory activity of unripe fruits was superior to that of ripe ones in accordance with the fact that the contents of four flavanone glycosides in unripe fruits were higher than those in ripe ones. Inhibitory activity of *C. hassaku* fruit was more potent than that of *C. unshiu* fruit. The 50% ethanolic extract (CH-ext) obtained from the unripe *C. hassaku* fruit collected in July exhibited significant tyrosinase inhibitory activity. Activity-guided fractionation and further examination revealed that the inhibitory activity of the CH-ext was attributable to two flavanone glycosides, naringin and neohesperidin. The tyrosinase inhibitory activities of naringin and neohesperidin are depicted in Table 8. Naringin showed the most potent activity. The SOD-like and anti-oxidant activities of CH-ext and its two flavanone glycosides were examined. As shown in Table 8, both CH-ext and neohesperidin showed potent SOD-like and DPPH radical-scavenging activities.

| Samples | Sampling month | $IC_{50}$ |
|---|---|---|
| CH-ext | July | 4.5 mg/ml |
| | August | 8.2 mg/ml |
| | September | >10 mg/ml |
| | October | >10 mg/ml |
| | November | >10 mg/ml |

Table 7. Seasonal variation in tyrosinase inhibitory activity ($IC_{50}$ value) of 50% ethanolic extract from *C. hassaku* fruits (*Ref.*; Itoh et al., 2009)

| Samples | Tyrosinase inhibitory activity | SOD-like activity | Radical-scavenging activity |
|---|---|---|---|
| CH-ext | 4.7 mg/ml | 0.5 mg/ml | 0.2 mg/ml |
| Naringin | 1.9 mM | >2000 μM | >4 mM |
| Neohesperidin | >5 mM | 26 μM | 0.6 mM |
| Narirutin | 2.0 mM | >2000 μM | >4 mM |
| Hesperidin | >5 mM | 268 μM | 3.2 mM |
| Arbutin | >10 mM | N.D. | N.D. |
| Kojic acid | 0.02 mM | N.D. | N.D. |
| Superoxide dismutase (SOD) | N.D. | 0.2 U/ml | N.D. |
| L-Ascorbic acid | N.D. | N.D. | 0.03 mM |

Table 8. $IC_{50}$ values of tyrosinase inhibitory, SOD-like, and radical-scavenging activities of 50% ethanolic extract from *C. hassaku* fruits (CH-ext), naringin, neohesperidin, narirutin, hesperidin, arbutin, kojic acid, superoxide dismutase, and L-ascorbic acid (N.D.: not determined. *Ref.*; Itoh et al., 2009)

The inhibitory effects of CH-ext on melanogenesis were evaluated (Itoh et al., 2009). The CH-ext showed significant inhibitory activity in a concentration-dependent manner without any significant effects on cell proliferation, as depicted in Table 9. Moreover, as for the pharmacological activity of CH-ext, we found anti-allergic, fibrinolytic, collagen-induced rabbit platelet aggregation inhibitory, and polybrene-induced rat erythrocyte aggregation inhibitory activities. These results imply that CH-ext can improve blood fluidity, which is related to skin problems such as infraorbital dark circles around the eyes and skin darkness resulting from unsmooth circulation or blood stagnation. Thus, CH-ext and its flavanone glycosides may be useful ingredients for whitening cosmetics.

| Samples | Concentration | Melanin content (μg/well) | Cell proliferation (%) |
|---|---|---|---|
| Control | | 13.3 ± 0.6 | 100.0 ± 5.9 |
| CH-ext | 100 μg/ml | 11.2 ± 0.6** | 113.3 ± 5.7 |
| | 250 μg/ml | 9.9 ± 0.5** | 122.2 ± 6.4 |
| | 500 μg/ml | 7.0 ± 0.2** | 101.8 ± 13.1 |
| Arbutin | 50 μM | 11.5 ± 0.1* | 84.6 ± 5.9 |
| | 100 μM | 11.6 ± 0.1* | 91.9 ± 7.6 |
| | 250 μM | 8.5 ± 0.2** | 103.3 ± 12.0 |
| | 500 μM | 6.5 ± 0.3** | 100.2 ± 8.8 |
| Kojic acid | 50 μM | 13.6 ± 1.0 | 100.9 ± 6.6 |
| | 100 μM | 13.2 ± 0.2 | 101.7 ± 11.1 |
| | 250 μM | 12.5 ± 0.6 | 116.4 ± 13.2 |
| | 500 μM | 10.6 ± 0.2** | 129.7 ± 10.1 |
| L-Ascorbic acid | 50 μM | 13.0 ± 0.4 | 120.7 ± 10.1 |
| | 100 μM | 15.4 ± 0.8** | 136.4 ± 15.2* |
| | 250 μM | 13.9 ± 0.8 | 134.1 ± 13.2 |
| | 500 μM | 14.9 ± 0.4* | 118.9 ± 15.1 |

Table 9. Effects of 50% ethanolic extract from unripe *C. hassaku* fruits (CH-ext) arbutin, kojic acid and L-ascorbic acid on melanin production in B16 melanoma cells (Each value represents the mean ± S.E. of triplicates. Statistical analysis was performed with a multiple comparison test using the Bonferroni/Dunn algorithm. Significantly different from the control group at *: $p<0.05$, **: $p<0.01$. *Ref.*; Itoh et al., 2009)

## 6. Conclusion

Research to find a novel inhibitor of melanin hyperpigmentation from natural resources was carried out based on our strategies. We found several seeds of melanogenesis regulators which exhibit various pharmacological actions. These seeds may become useful ingredients for cosmetics, supplements, functional foods and OTC-drugs.

Recent gradual destruction of the ozonosphere has raised solar UV exposure risk for all people. Solar UV radiation is a risk factor for photo-carcinogenesis, hyperpigmentation and photo-aging. Safer and more potent cosmetic whitening agents will be required to preserve beautiful and fair facial skin. We expect that superior melanin hyperpigmentation inhibitors with anti-aging effects and various anti-oxidative activities will be discovered from natural resources.

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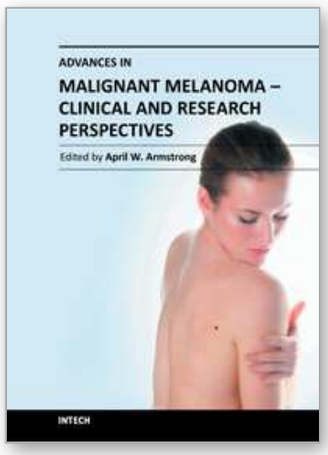


**Advances in Malignant Melanoma - Clinical and Research Perspectives**

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This book titled Advances in Malignant Melanoma - Clinical and Research Perspectives represents an international effort to highlight advances in our understanding of malignant melanoma from both clinical and research perspectives. The authors for this book consist of an international group of recognized leaders in melanoma research and patient care, and they share their unique perspectives regarding melanoma epidemiology, risk factors, diagnostic and prognostic tools, phenotypes, treatment, and future research directions. The book is divide into four sections: (1) Epidemiology and Risk Factors of Melanoma, (2) Clinical Phenotypes of Melanoma, (3) Investigational Treatments for Melanoma and Pigmentary Disorders, and (4) Advances in Melanoma Translational Research. This book does not attempt to exhaustively cover all aspects of the aforementioned topics. Rather, it is a compilation of our authorsâ€™ pearls and unique perspectives on the relevant advances in melanoma during the recent years.

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