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REVIEW ARTICLE

# Effect of quercetin derivatives on melanogenesis stimulation of melanoma cells

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**Abstract** Controlling melanogenesis is important for maintaining the good health and cosmetic appearance of a human body. This study aims to search the active compounds from natural products exhibiting the melanogenesis modulating activity and elucidate the mechanism underlying the observed activity. Two novel quercetin glycosides namely 4′-*O*-β-D-glucopyranosyl-quercetin-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranoside **1** and 4′-*O*-β-D–glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-quercetin-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranoside **2** were isolated and identified from *Helminthostachys zeylanica* roots 50 % ethanol extract. Compound **1** exhibited intracellular melanogenesis stimulatory activity, while **2** showed no effect even the structural similarity. To understand the structure–activity relationships, twelve quercetin glycosides and seven methylquercetins were synthesized from rutin as starting material. As the result of bioassay using synthesized nineteen quercetin derivatives in B16 melanoma cells, some quercetin-3-*O*-β-D-glucopyranosides stimulated the intracellular melanogenesis. On the other hand, synthesized 3-*O*-methylquercetin **12** and 3,4′,7-*O*-trimethylquercetin **15** increased both intra and extracellular melanin contents with no cytotoxicity. Compound **15** increased the phosphorylated p38 mitogen-activated protein kinase (MAPK) and microphthalmia-associated transcription factor (MITF) which regulates the expression of tyrosinase, TRP-1 and TRP-2. While **12** enhanced the expression of the melanogenic enzymes without involving the MITF, as evidenced by its lack of any stimulation of the expression of MITF and p-p38 MAPK. This result indicates that **12** may stimulate the expression of tyrosinase, TRP-1, and TRP-2 by stimulating currently unidentified transcriptional factors and/or by regulating the degradation of melanogenic enzymes.

**Keywords** *Helminthostachys zeylanica* · Quercetin glycoside · Melanin biosynthesis · Tyrosinase · p-p38 MAPK

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## Melanin biosynthesis

Melanin pigment is distributed in several tissues in the human body. The main function of melanin is considered to prevent skin damage by ultraviolet (UV) rays including in sunlight [1, 2]. The excess accumulation of melanin or the absence of melanin production caused by aging, strass, and UV damages induces gray hairs, freckles, mottling, and senile lentigines [3]. Hence, regulating melanogenesis is desired to maintain the good health and cosmetic appearance of the human body. Keratinocytes, existing on the skin surface, produce messengers such as α-melanocyte-stimulating hormone (α-MSH), prostaglandin, and histamine to melanocytes after stimulated by UV irradiation [4]. Then, melanocyte biosynthesizes the melanin in melanosome and transports the mature melanosomes to the keratinocytes. The skin pigmentation is induced by the cornification of keratinocyte including the mature melanosomes that are biosynthesized and transported by the melanocytes (Fig. 1). Similarly, hair pigmentation occurs due to melanin released

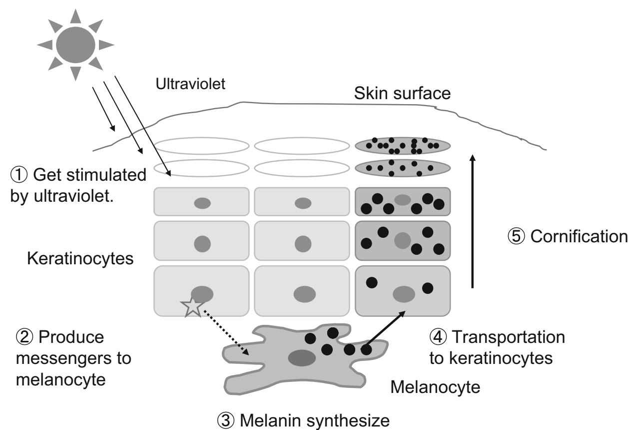



**Fig. 1** Mechanism of skin pigmentation

on the outside of the melanocyte. During active growth of hair follicles, melanocytes locate in the hair blub proliferated and differentiate to produce pigment of the hair shaft [5, 6]. Melanocytes begin to shut down melanogenesis in late anagen and regression phase called catagen, and it die by apoptosis on the hair bulb in rest phase called telogen. The melanogenesis in melanocytes reappears when hair follicles reenter anagen [7–10]. The prevention of melanogenesis in the hair bulb on anagen is induced by the aging or stress which results in gray hair.

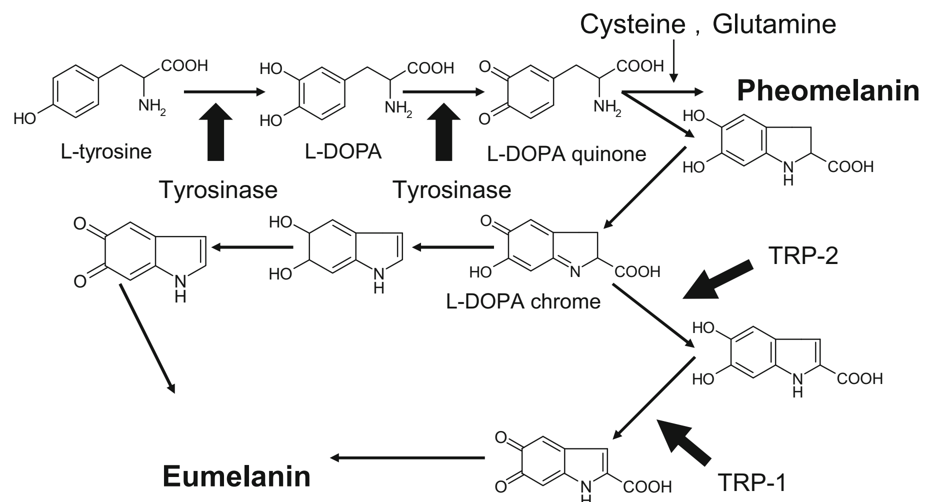
Melanin biosynthesis initially takes place via oxidation of L-tyrosine catalyzed by tyrosinase in melanosome, a rate limiting reaction of melanin biosynthesis (Fig. 2). Tyrosinase catalyzes the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), as well as the subsequent oxidation of L-DOPA to L-DOPA quinone [11, 12]. Tyrosinase contains two copper ions, and inactive tyrosinase is activated via taking in oxygen and $H_2O$. The active tyrosinase binds with tyrosine or L-DOPA and release the oxidized product via constitution of intermediate. Two types of melanin are ultimately biosynthesized, reddish-orange and blackish-brown pigments called pheomelanin and eumelanin, respectively, with the enzymes tyrosinase-related protein (TRP)-1 and TRP-2 playing a key role in the biosynthesis of eumelanin [13]. Then regulation of tyrosinase, TRP-1, and TRP-2 activity and/or expressions plays an important role in controlling melanin production.

## Transcriptional regulation of melanogenesis

The expressions of melanogenic enzymes, tyrosinase, TRP-1, and TRP-2, are transcriptionally regulated by microphthalmia-associated transcription factor (MITF) and several kinds of kinase pathway [14–17] as shown in Fig. 3. The amounts of cyclic adenosine monophosphate (cAMP) in melanocyte is increased by the messenger such as histamine, α-MSH, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and prostaglandin released by keratinocyte after get stimulated by UV [4, 18, 19]. The effects of messengers are induced via interaction with receptors on the melanocytes. Melanocortin 1 receptor (MC1R), a receptor of α-MSH, leads to elevation of cAMP contents by the interaction with α-MSH.

The increase of cAMP contents results in the regulation of the expression on protein kinase A (PKA), p38 mitogen-

**Fig. 2** Melanin biosynthesis pathway



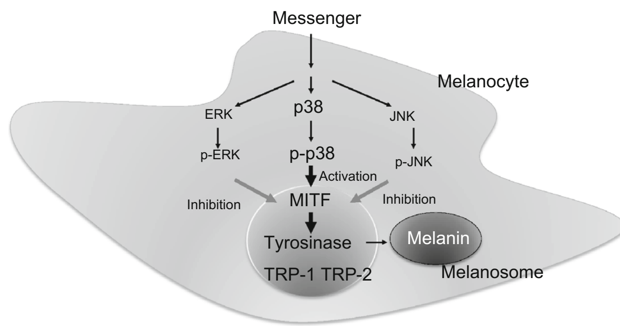



**Fig. 3** Transcriptional regulation of expression of melanogenic enzymes

activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) which regulate the expressions of melanogenic enzymes [20]. PKA phosphorylates the cAMP response element-binding protein (CREB), which is known to be an activator of MITF expression [16, 21–23]. Phosphorylated p38 MAPK stimulates the expression of melanogenic enzymes via activating the MITF expression [24, 25]. Additionally, p38 MAPK regulates melanogenesis by stimulating proteasomal degradation of melanogenic enzymes [26]. ERK phosphorylates more than 160 proteins, including transcription factors, enzymes, protein kinase relating to signal transduction, and so on, and it ultimately down regulates the expression of MITF and melanogenesis [27–30]. JNK, involved in proliferation and apoptosis cancer cells, also controls the melanogenesis by down-regulating the expression of MITF [31–33].

## Melanogenesis controlling effect of medicinal plants extract

Medicinal plants have been traditionally used to maintain good health and beauty on human body such as curing the inflammatory, preventing microbial infection, skin whitening, etc. Indonesian tropical forests, containing 28,000 plant species, cover about 143 million hectares and are home to about 80 % medicinal plants in the world. Jamu, an Indonesian traditional herbal medicine, have been generally used in Indonesia so far. For instance, a Jamu including *Trigonella foenum graecum*, *Tribulus terrestris*, *Yohimbe*, *Talinum paniculatum*, and *Plantago major* have been used to treat liver and kidney disturbance. *Orthosiphonis*, *Phyllanthi*, *Plantaginis*, *Blumeae*, *Centellae*, *Morindae*, *Alstoniae*, *Andrographidis,* and *Cercospora apii* have been used for treating mild hypertension, and *Morindae fructus*, *Orthosiphonis folium*, *Syzygii polyanthi*, *Andrographidis*, *Centellae*, and *Curcumae* have been used to treat diabetes mellitus [34].

To clarify the mechanism of the biological activity of Jamu, the biological activity of Indonesian medicinal plants extracts has been investigated, and isolation and identification of bioactive components including Indonesian medicinal plants have been performed in the world because of its valuable pharmaceutical potential. A number of bioactivity of Indonesian medicinal plants have been reported and novel compounds and bioactive components have been isolated from Indonesian herbal medicinal plants used as Jamu. Kamiya et al. [35] isolated five novel flavonoid glucuronides from fruit of *Helicteres isora* which is called Ulet–Ulet in Java island and used as Jamu in Indonesia. A novel anthraquinone glucoside was isolated from the root of *Rheum palmatum*, an Indonesian Jamu [36]. The extracts of traditional Indonesian medicinal plants, *Cinnamomum massoiae, Eucalyptus globulus, Vitex trifolia, Eucalyptus globulus, Plantago major* L., and *Vitex trifolia* L. inhibit the histamine release from rat basophilic leukemia cells [37]. In this study, the lead compounds exhibiting melanogenesis controlling effect was searched using *Helminthostachys zeylanica*, an Indonesian medicinal plant.

Traditional medicinal plants have been used for cosmetics especially for whitening agents. As described above, melanin is synthesized via rate limiting reaction by tyrosinase catalysis from L-tyrosine as a starting material. Hence a number of extracts of medicinal plants and the components have been treated to determine the tyrosinase inhibitory activity in order to search the whitening agents. Morin as shown in Fig. 4, one of the flavonol and widely distributed in plants including onion, guava leaves and seaweeds, exhibits potent tyrosinase inhibitory activity [38, 39]. Glabridin, an isoflavan derivatives, was isolated from *Allamanda cathartica* stem as potent tyrosinase inhibitor [40, 41]. Artocarbene, chlorophorin, norartocarpanone, and 4-propylresorcinol including 2,4-substituted resorcinol moiety induce high tyrosinase inhibitory activity [42, 43]. Catechol moiety of chalcone exhibits tyrosinase inhibitory activity, and it is reported that the catechol chelates with the copper ions, present in the active site of tyrosinase. While the 2, 4-substituted resorcinol shows no chelate with the copper ions and inhibits tyrosinase activity more potently than catechol by competitive binding with the copper ions in tyrosinase. The tyrosinase activity and melanogenesis of prenylated flavonoids from *Artocarpus altilis* were determined [44]. *A. altilis* is popularly known as the breadfruit tree in English. Besides the leaves, roots, and root bark are used as traditional medicines in West Indies to relieve asthma, decrease blood pressure, cure liver disorders, and decrease fever [45]. Norartocarpetin from *A. altilis* also including 2, 4-substituted resorcinol moiety exhibits potent tyrosinase inhibitory activity as well as melanogenesis inhibitory activity in B16 melanoma cells.

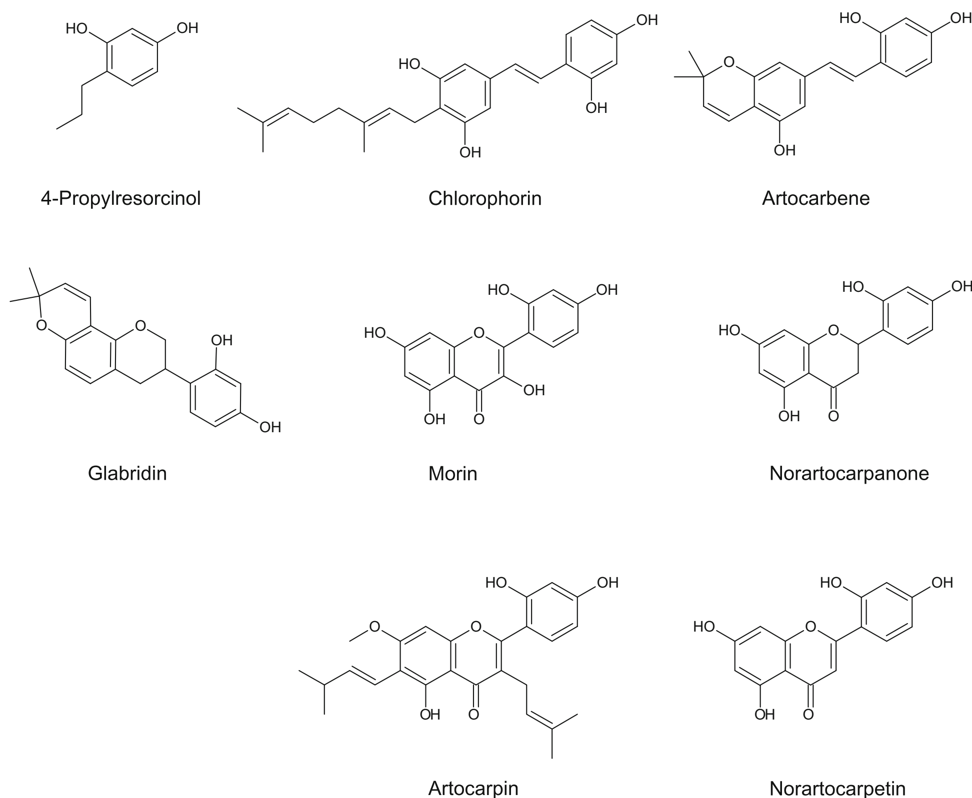



**Fig. 4** Chemical structures of tyrosinase inhibitor including 2, 4-substituted resorcinol moiety

While artocarpin which has 2, 4-substituted moiety shows less potent tyrosinase inhibitory activity than norartocarpetin because of its low polarity substituent groups. However, it should be noted that artocarpin exhibits higher melanogenesis inhibitory activity in melanoma cells than norartocarpetin even though artocarpin shows low tyrosinase inhibitory activity, suggesting that it is necessity to consider the expressions of melanogenic enzymes as well as tyrosinase activity in melanoma cells. As described above, a lot of tyrosinase inhibitors have been obtained and investigated the mechanism. However, there are few reports focusing on the compounds stimulating tyrosinase activity thus far.

## Regulator on expression of melanogenic enzymes

Tyrosinase is transcriptionally regulated by MITF, and MITF expression is regulated by a number of kinase pathways. Recently, the melanogenesis-regulating activity of compounds have been evaluated by determining the tyrosinase expression as well as tyrosinase activity. Besides to clarify the mechanism, the expressions of MITF and kinases have been investigated by western blot analysis. 1-*O*-Methyl-fructofuranose from the fruit of *Schisandra chinensis* is a traditional Korean medicinal herb, and the effects of 1-*O*-methyl-fructofuranose on melanogenesis, expressions of melanogenic enzymes, and related signaling pathways were investigated. 1-*O*-Methyl-fructofuranose inhibits melanogenesis by suppressing the expressions of tyrosinase, TRP-1, and MITF. Additionally, the study also elucidated the compound increases the phosphorylation of ERK, suggesting 1-*O*-methyl-fructofuranose inhibits melanogenesis by stimulating the ERK pathway [46]. Citrus fruits press cake was reported as melanogenesis inhibitor by suppressing MITF expression [47]. *Rhodiola rosea* extracts was studied as melanogenesis suppressor. The acetone extract of *R. rosea* exhibits tyrosinase inhibitory activity [48]. Besides *R. rosea* root containing phenylethanol derivatives, phenylpropanoids, monoterpenes, flavonoids, phenolic acids, and triterpenes [49, 50] decreases melanin content in B16 melanoma cells via suppressing the expressions of MC1R, a receptor of α-MSH, MITF, TRP-1, and tyrosianse [51].

On the other hand, melanogenesis stimulator also has been studied. Diethylstilbestrol was reported to exhibit potent melanogenesis stimulatory activity. Diethylstilbestrol increases the tyrosinase, TRP-1, TRP-2, and MITF mRNA as well as tyrosinase activity [52]. Cilostazol (6-[4-(1-cyclohexyl-1 Htetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinolinone), known as an inhibitor of cAMP-degrading enzyme, promotes melanogenesis by increasing the expressions of MITF via PKA/CREB pathway [53].

Recently a number of papers have focused on the modulation of tyrosinase activity and expressions of melanogenic enzymes to control the skin and hair pigmentation. The skin and hair pigmentation takes place by the melanosomes transported and released from melanocyte. However, few studies have treated the compounds which could control the transportation of melanosomes as well as the expressions of melanogenic enzymes. Several proteins, involved in the transportation of melanosomes, have been identified and a lot of the mechanism of the transportation has been already clarified. Therefore, the investigation of regulators on transportation of melanosomes is desired.

## Bioactivities of *H. zeylanica* extracts

To search the lead compounds modulating the melanogenesis activity, *H. zeylanica* (Fig. 5) root extract has been focused on because of the melanogenesis stimulatory activity on the screening assay. *H. zeylanica*, which belongs to Ophioglossaceae family, has been used for pain relief, germ killing, wound care, and promotion of bone healing after fracture [54, 55]. Besides it has been used as an antipyretic, antiphlogistic, and anodyne [56] and used to treat sciatica, boils, ulcers and malaria [57]. The bioactive components included in *H. zeylanica* have been isolated and identified so far. Four flavonoids, ugonins A–D were isolated from rhizomes [58, 59]. Moreover, ugonins E–T were isolated from root as antioxidant and anti-inflammatory activity by Huang [60, 61]. Additionally, ugonin K promoted osteoblastic differentiation and mineralization via activating of p38 MAPK and ERK pathway [54].

## Quercetin glycosides from *H. zeylanica* root as intracellular melanogenesis stimulator

Two novel quercetin glycosides compound **1** and **2** (Fig. 6) were isolated from a water/EtOH extract of *H. zeylanica* root via column chromatography, and identified as 4′-*O*-β-D-glucopyranosyl-quercetin-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranoside and 4′-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-quercetin-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranoside, respectively, by NMR and UPLC-TOFMS in our paper [62].

The intracellular melanogenesis enhancement activity of compound **1** and **2** at 10 μM of concentration was investigated. The intracellular melanogenesis activity and cell viability of compound **1** were 270 and 93 %, respectively, while compound **2** has no intracellular melanogenesis enhancement activity without affecting tyrosinase activities. Quercetin, the aglycon of compound **1** and **2**, has high intracellular melanogenesis inhibitory activity. However, quercetin-4′-*O*-β-D-glucoside, quercetin-3-*O*-β-D-glucoside, quercetin-3, 4′-*O*-β-D-glucoside, and rutin showed a lower inhibitory activity than quercetin [63]. However, quercetin-3-*O*-β-D-glucoside enhances melanogenesis by accelerating the expression of TRP-1 and -2 [64]. Our

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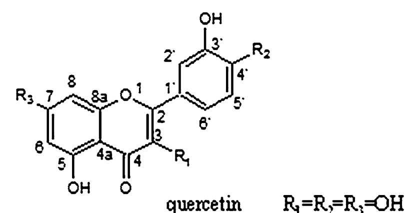
Courtesy of Katsunori Ueno

**Fig. 5** *Helminthostachys zeylanica*




Compound

| | | | |
|---|---|---|---|
| 1 | $R_1$=cellobiose, $R_2$=glucose, $R_3$=OH | | |
| 2 | $R_1$=cellobiose, $R_2$=sophorose, $R_3$=OH | 12 | $R_1=OCH_3$, $R_2=R_3=OH$ |
| 3 | $R_1$=glucose, $R_2=R_3$=OH | 13 | $R_1=R_2=OCH_3$, $R_3=OH$ |
| 4 | $R_1$=cellobiose, $R_2=R_3$=OH | 14 | $R_1=R_3=OCH_3$, $R_2=OH$ |
| 5 | $R_1$=cellobiose, $R_2$=OH, $R_3$=glucose | 15 | $R_1=R_2=R_3=OCH_3$ |
| 6 | $R_1$=cellobiose, $R_2$=cellobiose, $R_3$=OH | 16 | $R_2=OCH_3$, $R_1=R_3=OH$ |
| 7 | $R_1$=cellobiose, $R_2$=OH, $R_3$=cellobiose | 17 | $R_3=OCH_3$, $R_1=R_2=OH$ |
| 8 | $R_1$=glucose, $R_2$=glucose, $R_3$=OH | 18 | $R_1=OH$, $R_2=R_3=OCH_3$ |
| 9 | $R_1$=glucose, $R_2$=OH, $R_3$=glucose | 19 | $R_1$=OAc, $R_2=R_3$=OH |
| 10 | $R_1$=glucose, $R_2$=cellobiose, $R_3$=OH | 20 | $R_1$= tetraacetylglucose |
| 11 | $R_1$=glucose, $R_2$=OH, $R_3$=cellobiose | | |

**Fig. 6** Structures of quercetin derivatives

results showed the similar and curious tendencies that **1** has intracellular melanogenesis acceleration activity of 2.7 times to control, while interestingly compound **2** has no intracellular melanogenesis enhancement activity in spite of the similarity of the structure. This result means the number of sugar connecting C-4′ may play an important role in the melanogenesis activity.

Tyrosinase is transcriptionally regulated by MITF and its expression is activated by the p38 MAPK cascade. On the other hand, ERK and JNK pathway have been reported to be related to the down-regulation of melanin synthesis [64]. Some melanogenic enhancing agents have been examined at several points of melanogenesis such as expression of tyrosinase, p38, JNK, ERK and MITF as well as tyrosinase activity.

## Synthesis of quercetin derivatives and the melanogenesis stimulatory activity via p38 pathway

Quercetin is a flavonoid present as a glycoside in various fruits and vegetables [65–67]. A number of studies have demonstrated that quercetin exhibits a variety of pharmacological effects, including antioxidant and anti-cancer activities [68, 69], while some reports relate to effectiveness in controlling melanogenesis. Quercetin is recognized as a potent inhibitor of tyrosinase activity and melanogenesis, as evidenced by the studies performed in the mouse B16 melanoma cells [70]. However, quercetin has been reported to elicit the opposite effect and accelerate melanogenesis in human melanoma cells [71]. Furthermore, it was reported that the direction of its melanogenesis-regulating activity depends on the concentration of quercetin used [72]. A small number of studies have shown that quercetin derivatives can control melanogenesis. Quercetin-3-*O*-β-D-glucoside enhances melanogenesis by stimulating the expression of TRP-1 and TRP-2 [64].

Chemical synthesis of compounds isolated from natural products plays an important role to obtain the abundant amount of the compounds and to identify the exact chemical structure of the compounds. The synthesis strategy of quercetin derivatives has been studied so far because of their high potency on biological activity. For instance, quercetin 3-*O*-β-D-glucuronide was synthesized from rutin as a starting material [73]. Regiospecific synthesis of quercetin-*O*-β-D-glucosylated and quercetin-*O*-β-D-glucuronidated isomers using dichlorodiphenylmethane to protect hydroxyl groups of catechol moiety on B ring [74]. Additionally five *O*-monomethylated analogs of quercetin (3′-*O*-methylquercetin, 4′-*O*-methylquercetin, 3-*O*-methylquercetin, 5-*O*-methylquercetin, and 7-*O*-methylquercetin) were synthesized through sequential protection using dichlorodiphenylmethane [75].

We synthesized twenty quercetin derivatives as shown in Fig. 6 according to the previous papers to investigate the structure–activity relationships of quercetin derivatives on melanogenesis stimulatory activity.

The melanogenesis activities by adding the synthesized quercetin glycosides were determined with measuring intra and extracellular melanin content in B16 melanoma cells. The data for cell viability and the melanogenesis activity of B16 melanoma cells are shown in Table 1. Quercetin glycosides **1**, **3**, and **4** showed intracellular melanogenesis stimulatory activity in a dose-dependent manner, and their activities were higher than that of theophyline used as positive control [76]. Interestingly, the other quercetin glycosides had little or no melanogenesis stimulatory activity despite their structural similarities. The molecules with 7-*O*-glycoside showed no intracellular melanogenesis stimulatory activity. On the other hand, the molecules with 3-*O*-glucoside, with a free OH group on the B ring, and the molecules with 3-*O*-cellobioside, with a free OH group or 4′-*O*-glucoside showed intracellular melanogenesis stimulatory activity. Thus, a hydroxyl group on C-7 may play an important role in melanogenesis activity. Additionally, the chemical structure of the sugar connected at the C-4′ may also play an important role in the activity. Moreover, it should be noted that the opposite effect was reported that quercetin and quercetin-4′-*O*-glucoside which have the hydroxyl group at C-3 inhibited the melanin biosynthesis [77]. These results indicated that the hydroxyl group at C-3 of quercetin derivatives may be important to suppress the melaninogenesis activity, and the effect for melanin biosynthesis in B16 melanoma cells of quercetin glycosides are varied significantly by the presence or absence of hydroxyl group especially combing to C-3 or C-7 position. Furthermore, the activity may be more complex by the kind, size or polarity of the sugars connecting to the quercetin, therefore it is necessity the further investigations to elucidate completely the structure–activity relationships of quercetin glycosides.

The effects of compounds **12–20** on cell viability and melanogenesis are shown in Table 2. We evaluated the modulation of intra and extracellular melanin levels by quercetin methylethers with theophylline as a positive control. On measuring the melanogenesis activity assay for each compounds, we adopted a concentration which was not shown strong cytotoxicity of the B16 melanoma cells. As shown in Table 1, quercetin glycosides **1**, **3**, and **4** stimulated intracellular melanogenesis in a dose-dependent manner. However, none of the synthesized quercetin glycosides increased the extracellular levels of melanin. On the other hand, quercetin methylethers **12–15** increased both intra- and extracellular melanin content (Table 2), demonstrating higher melanogenesis stimulation than theophylline, a positive control. Significant effects were

**Table 1** Intra- and extracellular melanogenesis activity and cell viability in B16 melanoma cells by the synthesized quercetin glycosides **1–11**

| | Cell viability and melanogenesis activity (%) | | |
|---|---|---|---|
| | 200 μM | 100 μM | 10 μM |
| **1** | | | |
| Intercellular melanogenesis activity | 190.5 ± 10.2* | 151.5 ± 8.1* | 137.0 ± 0.8* |
| Extracellular melanogenesis activity | 125.5 ± 9.3 | 116.0 ± 12.6 | 99.7 ± 21.7 |
| Cell viability | 74.8 ± 2.1 | 86.8 ± 2.0 | 90.0 ± 6.9 |
| **3** | | | |
| Intercellular melanogenesis activity | 206.9 ± 2.3** | 157.1 ± 8.6* | 116.3 ± 1.3 |
| Extracellular melanogenesis activity | 86.1 ± 2.7 | 85.1 ± 1.7 | 91.0 ± 4.1 |
| Cell viability | 68.4 ± 6.9* | 80.9 ± 5.5 | 105.0 ± 3.8 |
| **4** | | | |
| Intercellular melanogenesis activity | 176.0 ± 9.1* | 126.8 ± 9.1* | 102.6 ± 2.9 |
| Extracellular melanogenesis activity | 85.2 ± 4.9 | 91.0 ± 6.3 | 83.3 ± 5.8 |
| Cell viability | 81.1 ± 3.0 | 93.2 ± 0.6 | 99.6 ± 1.5 |
| **5** | | | |
| Intercellular melanogenesis activity | 119.0 ± 7.4 | 78.9 ± 6.0 | 91.1 ± 5.5 |
| Extracellular melanogenesis activity | 93.3 ± 6.4 | 105.8 ± 3.3 | 102.4 ± 2.8 |
| Cell viability | 98.7 ± 12.4 | 112.6 ± 2.6 | 95.1 ± 1.6 |
| **6** | | | |
| Intercellular melanogenesis activity | 99.7 ± 1.8 | 84.77 ± 2.1 | 79.7 ± 4.2 |
| Extracellular melanogenesis activity | 80.9 ± 3.8 | 92.3 ± 2.7 | 91.2 ± 2.0 |
| Cell viability | 99.7 ± 1.8 | 106.0 ± 8.3 | 106.8 ± 3.4 |
| **7** | | | |
| Intercellular melanogenesis activity | 102.6 ± 15.5 | 97.8 ± 7.2 | 80.3 ± 2.14 |
| Extracellular melanogenesis activity | 96.9 ± 5.4 | 102.1 ± 0.6 | 93.0 ± 1.2 |
| Cell viability | 95.2 ± 0.4 | 93.9 ± 7.6 | 105.3 ± 0.7 |
| **8** | | | |
| Intercellular melanogenesis activity | 92.6 ± 0.0 | 90.4 ± 15.1 | 89.7 ± 11.9 |
| Extracellular melanogenesis activity | 103.2 ± 11.7 | 95.0 ± 2.3 | 100.0 ± 3.5 |
| Cell viability | 77.4 ± 10.4 | 104.1 ± 3.5 | 107.3 ± 6.5 |
| **9** | | | |
| Intercellular melanogenesis activity | 86.5 ± 9.1 | 113.9 ± 14.4 | 85.0 ± 1.5 |
| Extracellular melanogenesis activity | 74.6 ± 9.3 | 113.7 ± 0.5 | 103.2 ± 0.1 |
| Cell viability | 114.4 ± 4.8 | 78.0 ± 1.3 | 97.7 ± 2.5 |
| **10** | | | |
| Intercellular melanognenesis activity | 96.3 ± 0.9 | 90.9 ± 13.3 | 96.8 ± 7.6 |
| Extracellular melanogenesis activity | 87.3 ± 12.6 | 95.1 ± 5.8 | 99.9 ± 3.8 |
| Cell viability | 105.6 ± 6.4 | 107.0 ± 2.6 | 97.8 ± 8.9 |
| **11** | | | |
| Intercellular melanogenesis activity | 83.8 ± 1.9 | 78.9 ± 6.0 | 91.1 ± 5.5 |
| Extracellular melanogenesis activity | 91.9 ± 18.0 | 90.1 ± 2.1 | 104.5 ± 7.1 |
| Cell viability | 111.0 ± 9.6 | 112.6 ± 2.6 | 95.1 ± 1.6 |
| Theophyline | 500 μM | 250 μM | 125 μM |
| Intercellular melanogenesis activity | 166.8 ± 31.7* | 131.7 ± 1.9 | 127.6 ± 6.6 |
| Extracellular melanogenesis activity | 204 ± 1.6** | 183.2 ± 3.2** | 170.7 ± 0.7** |
| Cell viability | 91.4 ± 1.3 | 94.4 ± 4.1 | 95.5 ± 11.0 |

Data are expressed as means ± SD ($n = 2$)

* $p \leq 0.05$ and ** $p \leq 0.01$ compared with respective control values

**Table 2** Intra- and extracellular melanogenesis activity and cell viability in B16 melanoma cells by the synthesized quercetin derivatives **12–20**

| | Cell viability and melanogenesis activity (%) | | | |
|---|---|---|---|---|
| | 50 μM | 25 μM | 12.5 μM | 6.25 μM |
| **12** | | | | |
| Intercellular melanogenesis activity | 157.3 ± 8.4* | 146.5 ± 15.3 | 137.0 ± 23.6 | – |
| Extracellular melanogenesis activity | 224.9 ± 18.2* | 130.8 ± 5.8* | 124.1 ± 1.4 | – |
| Cell viability | 74.4 ± 3.8 | 88.1 ± 4.1 | 98.2 ± 6.7 | – |
| **13** | | | | |
| Intercellular melanogenesis activity | – | 166.6 ± 0.0** | 178.8 ± 9.6** | 132.3 ± 8.1* |
| Extracellular melanogenesis activity | – | 346.7 ± 2.9** | 309.5 ± 14.5** | 229.5 ± 17.6* |
| Cell viability | – | 63.2 ± 2.1** | 50.0 ± 0.5** | 75.6 ± 2.9** |
| **14** | | | | |
| Intercellular melanogenesis activity | – | 187.6 ± 2.5** | 171.2 ± 0.0** | 134.0 ± 4.3* |
| Extracellular melanogenesis activity | – | 265.5 ± 5.9** | 304.3 ± 4.0** | 222.8 ± 12.8** |
| Cell viability | – | 54.1 ± 0.55** | 60.4 ± 4.2** | 80.4 ± 0.5** |
| **15** | | | | |
| Intercellular melanogenesis activity | – | 203.4 ± 3.4** | 181.4 ± 9.0** | 127.7 ± 4.3* |
| Extracellular melanogenesis activity | – | 298.7 ± 3.7** | 228.0 ± 7.0** | 225.5 ± 10.8** |
| Cell viability | – | 90.2 ± 4.3 | 101.9 ± 2.2 | 95.3 ± 1.8 |
| **16** | | | | |
| Intercellular melanogenesis activity | – | 106.9 ± 7.1 | 133.5 ± 4.4 | 125.6 ± 5.7 |
| Extracellular melanogenesis activity | – | 97.9 ± 0.0 | 95.1 ± 0.2 | 97.5 ± 2.2 |
| Cell viability | – | 100.9 ± 1.3 | 103.0 ± 4.3 | 103.6 ± 3.6 |
| **17** | | | | |
| Intercellular melanogenesis activity | – | 75.8 ± 12.9 | 87.6 ± 2.6 | 102.4 ± 12.0 |
| Extracellular melanogenesis activity | – | 114.3 ± 1.1 | 105.2 ± 1.3 | 105.5 ± 1.4 |
| Cell viability | – | 91.6 ± 4.5 | 99.4 ± 5.1 | 92.7 ± 0.5 |
| **18** | | | | |
| Intercellular melanogenesis activity | – | 100.5 ± 10.0 | 106.1 ± 8.1 | 110.2 ± 2.5 |
| Extracellular melanogenesis activity | – | 100.1 ± 0.3 | 98.0 ± 0.0 | 100.5 ± 0.5 |
| Cell viability | – | 101.3 ± 3.7 | 101.4 ± 1.9 | 99.7 ± 1.2 |
| **19** | | | | |
| Intercellular melanogenesis activity | 104.6 ± 3.1 | 114.3 ± 3.6 | 99.0 ± 8.3 | – |
| Extracellular melanogenesis activity | 126.6 ± 2.3 | 102.6 ± 6.2 | 87.6 ± 5.1 | – |
| Cell viability | 110.0 ± 7.9 | 120.4 ± 0.3 | 110.0 ± 3.8 | – |
| **20** | | | | |
| Intercellular melanogenesis activity | 148.4 ± 1.4** | 121.4 ± 17.1 | 122.4 ± 2.1 | – |
| Extracellular melanogenesis activity | 96.1 ± 1.8 | 101.5 ± 1.4 | 89.3 ± 0.4 | – |
| Cell viability | 103.7 ± 1.1 | 101.8 ± 0.2 | 113.5 ± 8.1 | – |

| Theophyline | 500 μM | 250 μM | 125 μM |
|---|---|---|---|
| Intercellular melanogenesis activity | 166.8 ± 31.7* | 131.7 ± 1.9 | 127.6 ± 6.6 |
| Extracellular melanogenesis activity | 204.0 ± 1.6** | 183.2 ± 3.2** | 170.7 ± 0.7** |
| Cell viability | 91.4 ± 1.3 | 94.4 ± 4.1 | 95.5 ± 11.0 |

Data are expressed as means ± SD ($n = 2$)

–, not done

* $p \leq 0.05$ and ** $p \leq 0.01$ compared with respective control values

observed on the extracellular melanin levels. Comparing the activities of compounds **12–15**, medium of cells incubated with 50 μM of compound **12** showed 224.9 % higher extracellular melanin levels compared to controls. The increases of melanin levels following incubation with compounds **13–15** were higher than 220 %, even at 6.25 μM, indicating the most potent stimulation of extracellular melanin levels in this study. Furthermore, the 3-hydroxyl quercetin methylethers such as **16–18**, 3-*O*-acethyl-quercetin **19**, and quercetin-3-*O*-β-D-2,3,4,6-tetra-*O*-acetoglucopylanoside **20** showed no stimulatory effect on the extracellular melanin levels, suggesting that the 3-methoxyl group of compounds **12–15** is an essential moiety for stimulation activity. Additionally, the 4′ and/or 7-methoxyl group may further increase the melanogenesis-stimulating activity. Compounds **13–15** showed more potent melanogenesis-stimulating activity compared to **12**. Importantly, **13** and **14** were associated with high cell cytotoxicity in the cell viability studies, while **15** exhibited high cell viability. These differences in cell cytotoxicity between the quercetin methylethers may depend on the presence of both 4′ and 7-methoxyl groups. These results are described in the previous published [78].

To understand the involvement of the tyrosinase enzyme in the stimulation of melanogenesis, the activity of mushroom tyrosinase was measured following incubation with quercetin derivatives. However, no effect on tyrosinase activity was observed with any of the quercetin derivatives synthesized in this study (data not shown). Therefore, the quercetin methylethers may contribute to the expression of tyrosinase or related genes in B16 melanoma cells.

## Effect of compounds 12 and 15 on the expression of proteins involved in melanin biosynthesis

In general, the signaling pathway modulating the expression of melanogenic enzymes in melanoma cells comprises the following steps. First, extracellular messengers, such as α-MSH and histamine, interact with receptors on the melanocyte. Receptor stimulation increases intracellular cAMP levels in the melanocyte, stimulating a number of intracellular kinase pathways, such as the p38 MAPK, ERK, and JNK. These kinase signaling cascades regulate the expression of MITF, which acts as a transcriptional factor regulating the expression of tyrosinase, TRP-1, and TRP-2.

Some studies have reported that melanogenesis-modulating agents regulate the expression of tyrosinase, TRP-1, and TRP-2 by regulating the expression of p38 MAPK, ERK, JNK, and MITF. For example, the components isolated from *Nardostachys chinensis* and *Rhodiola rosea* crude extracts were reported to inhibit melanin biosynthesis by suppressing the expression of MITF and tyrosinase in B16 melanoma cells. Similarly, the compounds showing melanogenesis-stimulating activity in this study may also control the levels of tyrosinase and the proteins that modulate its expression, as suggested by the observation that the stimulatory activity does not depend on the tyrosinase activity.

As presented in Table 2, compound **15** showed significant intra- and extracellular melanogenesis-stimulating activity, with low cytotoxicity. The effects of compound **15** on the expressions of proteins related to melanin biosynthesis, such as tyrosinase, TRP-1, TRP-2, MITF, p-p38 MAPK, and p38 MAPK were investigated to identify the specific biosynthetic step associated with its activity. As shown in Fig. 7, **15** increased the expression of tyrosinase, TRP-1, TRP-2, MITF, and p-p38 MAPK in a dose-dependent manner in B16 melanoma cells. Conversely, the expression of p38 MAPK was not increased by **15**, indicating that it stimulates melanin biosynthesis by stimulating the p38 MAPK phosphorylation. Furthermore, the melanogenesis-stimulating effects of **12** were determined to compare the activity on the expression of the proteins. Comparing the activities of **12** and **15** on the expression of proteins related to melanin biosynthesis, **12** was found to increase the expression ratio of the tyrosinase and TRP-1 to a greater extent than **15**. Nevertheless, extracellular melanogenesis-stimulating activity of **12** was lower than that of **15** (Table 2), suggesting that melanogenesis in melanoma cells is not solely dependent on the expression of tyrosinase and TRP-1. Furthermore, **15** significantly stimulated the expression of MITF and p-p38 MAPK, which enhance the expression of tyrosinase, TRP-1, and TRP-2. Conversely, **12** did not alter the expression of MITF and p-p38 MAPK, despite enhancing the expression of tyrosinase, TRP-1, and TRP-2. Except for MITF, no transcriptional factors controlling tyrosinase expression have been reported thus far. These results may therefore indicate that **12** enhances the expression of tyrosinase, TRP-1, and TRP-2 by stimulating transcriptional factors that are yet to be identified. The levels of melanogenic enzymes in the melanocyte are also regulated by protein degradation by proteasome through ubiquitination [26]. Fatty acids, a major component of the cell membranes, were previously reported to regulate melanin biosynthesis by controlling the degradation of tyrosinase, TRP-1, and TRP-2 [79]. Therefore, in addition to the possible effect on a transcriptional factor, an alternative explanation for the enhancing activity of **12** on the levels of melanogenic enzymes may involve the inhibition of the degradation of melanogenic enzymes through ubiquination. Compared to **15**, **12** increased the expression of melanogenic enzymes, but showed less melanogenesis-stimulating activity, as described above. Additionally, the levels of p-p38 MAPK

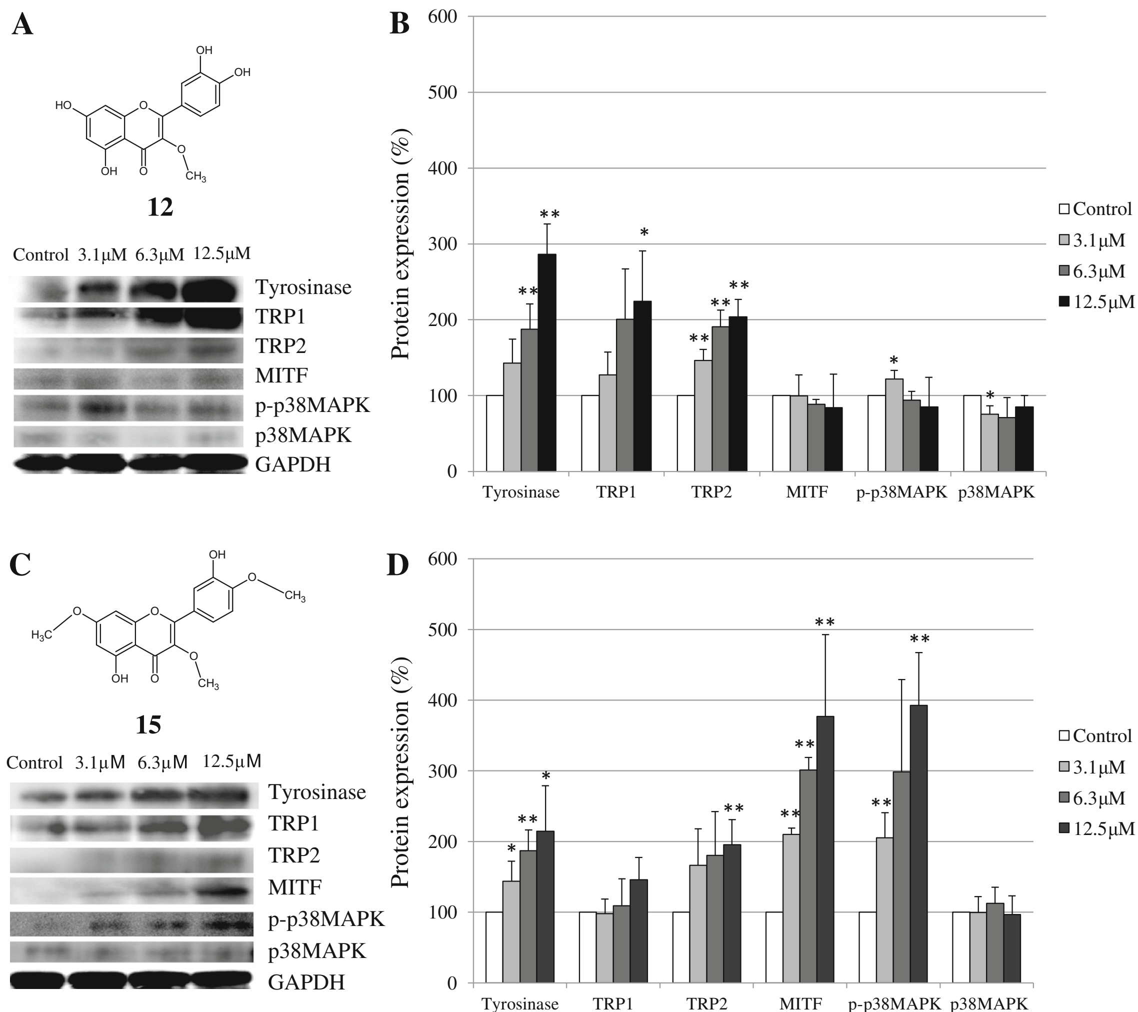



**Fig. 7** Effect of **12** and **15** on the expression of Tyrosinase, TRP1, TRP2, MITF, p-p38MAPK, and p38MAPK in B16 melanoma cells. **a** Representative blots of **12**. **b** Quantification of the ratio of protein expressions in melanoma cells treated by **12**. The data show the mean ± SD from three independent experiments. **c** Representative blots of **15**. **d** Quantification of the ratio of protein expressions in melanoma cells treated by **15**. The data show the mean ± SD from three independent experiments. *$p \leq 0.05$ and **$p \leq 0.01$ compared with control values

and MITF were not increased by the addition of **12**. If the two phenomena were related to each other, MITF and/or p-p38 MAPK may play an important role in the regulation of melanogenesis not only by enhancing the expressions of the enzymes, but also through affecting other factors, such as the transportation and/or degradation of melanogenic enzymes.

Tyrosinase, TRP-1, and TRP-2 are expressed through a MITF-regulated process and transported to the melanosome. Melanin is biosynthesized in the melanosome by the action of the melanogenic enzymes. The mature melanosome is specifically transported to the periphery of the cell from the perinuclear region of the melanocytes through a process that involves a wide variety of cellular transport proteins, including actin, myosin Va, Rab27A, and Slac2-a. Compound **15** may accelerate the transportation of melanogenic enzymes to the melanosome and/or transportation of melanosome to the outside of the cells by regulating the expression of proteins related to cellular transport. However, **12** may elicit less potent stimulation of the transportation of melanogenic enzymes to the melanosome and/or the transportation of melanosome as compared to **15**.

Among the nineteen synthesized compounds, **1**, **3**, and **4** are quercetin glycosides exhibiting intracellular melanogenesis stimulatory activity, while they showed no effect on extracellular melanogenesis. On the other hand, **12** and **15** increased both intra and extracellular melanin contents more potently than the positive control theophylline, with exhibiting low cytotoxicity. Compound **12** exhibited less melanogenesis-stimulating activity than compound **15**. However, **12** increased the expression of tyrosinase and

TRP-1 to a greater extent than **15**, thereby suggesting that melanogenesis in melanoma cells does not depend solely on the expression of the enzymes catalyzing melanin biosynthesis. Furthermore, **15** also stimulated the expression of the MITF and p-p38 MAPK, while they were not increased by **12**. These results suggest that **12** may enhance the expression of tyrosinase and TRP-1 by regulating the proteasomal degradation of melanogenic enzymes and/or by activating other transcriptional factors regulating enzyme expression.

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