

## Comparative study on melanin production and collagen expression profile of polyphenols and their glycosides

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A total of twenty different polyphenol aglycones and their biochemically synthesized glycoside derivatives were accessed for cell toxicity, collagen synthesis and melanin content inhibition assays to illustrate the double-edged sword role of glycobiology, in particular, modification of hydroxyl group in polyphenol structure without glycone moiety over anti-aging and defense to oxidative stress in skin dermatology. All molecules at (0.1-200)  $\mu\text{M}$  concentration inhibited cell growth in a dose dependent manner on human dermal fibroblast (HDF) and melanoma skin cancer (B16F10) cells. At lower concentrations of (0.1-10)  $\mu\text{M}$ , most of the molecules were nontoxic to HDF cells, while the same molecules were toxic to B16F10 cells except astilbin (6), baicalein (13), baicalein 7-O- $\beta$ -D-glucoside (14) and mangostin (18). Results showed that two molecules, quercetin (1) and diosmin (17), inhibited melanogenesis in  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH)-stimulated melanoma skin cancer cells (B16F10) in comparison to the control at 0.1  $\mu\text{M}$  concentration, indicating the possible use of these molecules in skin-whitening products. Similarly, the maintenance of collagen in HDF cells was found to be highly activated by the compound, kaempferol (11), at 1.0  $\mu\text{M}$  concentration, at which the cell viability was above 95%. Compound 1, apigenin 7-O- $\beta$ -D-glucoside (10) and baicalein (13) exhibited comparable collagen biosynthesis activity to control with significantly low cell toxicity.

**Keywords:** Anti-aging, Cytotoxicity, Flavonoid, Glycosides, Whitening

Plant derived polyphenols such as flavonoids are known for their anti-oxidant properties as they scavenge radicals generated in plants and animals. Thus, these molecules have wide application in human, animal and plant health, in terms of physiology and biochemical functions. Although several thousands of flavonoids and their derivatives have been isolated from plants, different biotechnological tools have been applied to generate flavonoid scaffolds in microbial systems, facilitating the further chemical diversity of natural and non-natural derivatives using post-modifying enzymes from different sources<sup>1</sup>. In general, flavonoids are modified to glycosides and stored in plants. Such modifications enhance the water solubility, temperature, pH stability, adsorption, distribution, metabolism and excretion processes of lead molecules. Thus, wide interest has been given to such molecules in industries for their use as potential pharmaceuticals, nutraceuticals and cosmetics.

Human skin is highly prone to exposure to different ranges of UV radiation, which generates reactive radicals, leading to destructive histochemical changes in the dermal cells<sup>2</sup>. Such skin ailments can be reduced by applying different cosmetic products that reinforce the beauty, longevity and attractiveness of skin<sup>3</sup>.

Hyper pigmentation (*i.e.* melanin over-production in human skin) is escalated by reactive radicals generated by exposure to UV radiation, resulting in change in skin color, skin wrinkling, aging and skin diseases such as freckles and chloasma dermatitis<sup>4</sup>. Anti-oxidants and radical scavengers are thus applied to suppress melanogenesis in the skin. Nevertheless, the radicals also damage the cutaneous proteins such as collagen, elastin and glycosaminoglycans, resulting in premature skin aging, wrinkle formations, laxity, elastosis, *etc.*<sup>5-7</sup>. Since collagen is the main structural unit of the extracellular matrix, type I collagen maintains the structural integrity of the skin. UV damages *de novo* type I collagen biosynthesis results in photo aging and wrinkle formation. Thus, the prevention of collagen degradation using radical scavengers and antioxidants is essential *via* nutrition, medicines or cosmetics.

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In this study, twenty different plant derived polyphenols and polyphenolic glycosides were assessed for their potential applications in skin care functions such as whitening and anti-aging *in vitro* using human dermal fibroblast (HDF) and melanoma skin cancer (B16F10) cells. *In vitro* studies of molecules is a cost-effective, fast and convenient approach for initial assessments of molecules to evaluate their efficacy on possible formulations in cosmetics.

## Materials and Methods

### Chemicals and reagents

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and  $\alpha$ -Melanocyte-stimulating hormone ( $\alpha$ -MSH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Flavonoids such as, quercetin, rutin, apigenin, myricetin, kaempferol, baicalein, baicalin, diosmin, hesperetin and  $\alpha$ -mangostin were purchased from Tokyo Chemical Industry (TCI) (Tokyo, Japan) and astilbin, diosmetin, hesperidin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other remaining flavonoid glycosides were produced in the laboratory using engineered microbial systems. Quercetin 3-O- $\alpha$ -L-rhamnoside<sup>8</sup>, quercetin 3-O- $\beta$ -D-glucoside<sup>9</sup>, quercetin 3-O- $\alpha$ -D-xyloside<sup>10</sup>, apigenin 7-O- $\beta$ -D-glucoside<sup>11</sup>, myricetin 3-O- $\alpha$ -L-rhamnoside<sup>12</sup>, kaempferol 3-O- $\alpha$ -L-rhamnoside<sup>8</sup> and baicalein 7-O- $\beta$ -D-glucoside<sup>11</sup> were biosynthesized in the laboratory by biotransformation using engineered *E. coli* cells. The structural identification of these molecules is presented in respective reports. All the molecules were purified using preparative high-performance liquid chromatography at a purity of up to 98%. Details of the molecules used in this study are given in (Table 1 and Suppl. Table 1)<sup>13-33</sup>.

### Cells and culture conditions

Human dermal fibroblasts (HDF) (ATCC # PCS-201-012) and melanoma skin cancer cells B16F10 (ATCC #CRL-6475) were used for the assay. Both cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). All cells were maintained at 37°C in a humidified 5% CO<sub>2</sub> incubator.

### Cell viability assay

The cytotoxicity of various compounds and their glycosylated derivatives were assessed using MTT microculture tetrazolium viability assay<sup>34</sup>. Precultured human dermal fibroblast (HDF) and B16F10 cells

( $2 \times 10^5$  cells/well) in DMEM medium were plated on a 96-well microplate. Different concentrations (0.1, 1.0, 10.0, 100 and 200)  $\mu$ M of the compounds were added to the cultured cells and incubated at 37°C under humidified 5% CO<sub>2</sub> condition for 24 h. After 24 h of incubation, 20  $\mu$ L of MTT (5 mg/mL) was added to each well and the plates were further incubated for 4 h. After the removal of the medium, 200  $\mu$ L dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The absorbance was then read at 595 nm using a microplate reader (Molecular Devices Co., CA, USA).

### Determination of melanin content

The melanin content of B16F10 melanoma cells was measured in the presence of various flavonoids and their glycosides. To determine the melanin content in the cell, B16F10 cells ( $2 \times 10^5$  cells/well) were seeded on a 96-well plate in Dulbecco's modified Eagle's medium supplemented with 10%

Table 1 — Details of compounds used in this study along with their structure

No	Name	Common Name	Class	Sugar(s)
1	Quercetin	—	Flavonol	—
2	Quercetin 3-O- $\alpha$ -L-rhamnoside	Quercitrin	Flavonol	Rhamnose
3	Quercetin 3-O- $\beta$ -D-glucoside	Isoquercetin	Flavonol	Glucose
4	Quercetin 3-O- $\alpha$ -D-xyloside	Reynoutrin	Flavonol	Xylose
5	Quercetin 3-O-rutinoside	Rutin	Flavonol	Glucose-rhamnose
6	Taxifolin 3-O-rhamnoside	Astilbin	Flavanonol	Rhamnose
7	Myricetin	—	Flavonol	—
8	Myricetin 3-O- $\alpha$ -L-rhamnoside	Myricitrin	Flavonol	Rhamnose
9	Apigenin	—	Flavone	—
10	Apigenin 7-O- $\beta$ -D-glucoside	Apigetrin	Flavone	Glucose
11	Kaempferol	—	Flavonol	—
12	Kaempferol 3-O- $\alpha$ -L-rhamnoside	—	Flavonol	Rhamnose
13	Baicalein	—	Flavone	—
14	Baicalein 7-O- $\beta$ -D-glucoside	—	Flavone	Glucose
15	Baicalein 7-O-glucuronide	Baicalin	Flavone	Glucuronic acid
16	Diosmetin	—	Flavone	—
17	Diosmetin 7-O-rutinoside	Diosmin	Flavone	Glucose-rhamnose
18	Mangostin	—	Xanthonoid	—
19	Hesperetin	—	Flavone	—
20	Hesperetin 7-O-rutinoside	Hesperidin	Flavone	Glucose-rhamnose

FBS and then incubated for 24 h. The cells were treated with 100 nm of  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) and flavonoid compounds (100 nm) and were then incubated for 24 h. The cells were harvested by centrifugation, then lysed using 100  $\mu$ L of 1N NaOH and then heated at 100°C for 30 min. The samples were transferred to a 96 well plate and the absorbance was measured at 415 nm using a microplate reader.

#### Quantitative RT-PCR (qRT-PCR) of *col1A1* gene

To evaluate the mRNA expression level of collagen type 1  $\alpha$  1 (*col1A1*) gene, the HDF cells were cultured in DMEM medium and incubated for 24 h at 37°C in a 96-well plate for the total mRNA extraction. After 24 h incubation, the cells were treated with flavonoids (0.1  $\mu$ M and 100  $\mu$ M) and incubated for a further 24 h under the same condition.

The cells were harvested and mRNA was extracted using TRIzol<sup>®</sup> reagent (Invitrogen) following the manufacturer's protocol. The purity of the isolated mRNA was determined by spectroscopic analysis at 260 nm and 280 nm. The total mRNA samples were stored at -80°C for further use. cDNA was prepared using reverse transcriptase enzyme. The qRT-PCR of the *col1A1* gene was performed in a PCR tube using HOT FIREPol Eva Green PCR Mix Plus (Solis BioDyne, Estonia), 1 pmole forward primer, 1 pmole reverse primer and 10 ng cDNA reaction mixture. The primers used for the *col1A1* gene were as follows: forward primer 5'-CGGTGTGACTCGTGACG-3' and reverse primer 5'-ACAGCCGCTTCACCTACAGC-3'.

The PCR results were verified using the melting curves of the individual PCR results. The analyses were conducted by normalizing the threshold cycle (*Ct*) values of individual genes to the *Ct* value of  $\beta$ -actin. The differences in the amount of the *Ct* values were then compared. Eva Green<sup>®</sup> dye (SYBR<sup>®</sup> safe DNA stain) was used to stain the PCR products to emit fluorescent light for the qRT-PCR analysis.

The *Ct* value is the number of cycles when the amount of fluorescent light (the number of amplified PCR products) generated by the PCR product reaches a certain criterion value that is equal to or higher than the basic value and the gene expression amount can be confirmed using the *Ct* value.

#### Statistical analysis

The student's *t*-test was performed on the biological replicate to determine the statistical significance of the difference between the control and

experiment samples at each time point for all the data. Differences with *P* value <0.05 were considered statistically significant. Biological activity results are expressed as the means  $\pm$  SE.

#### Results

Twenty different polyphenolic molecules were selected for the comparative analysis of potential skin care application. The molecules include aglycones and their different glycoside derivatives (Table 1). Quercetin (1) and its four different glycoside derivatives (different sugars conjugated at 3-OH position; 2, 3, 4, 5), astilbin (taxifolin 3-O-rhamnoside; 6), myricetin (7) and its 3-O-rhamnoside derivative (8), apigenin (9) and its 7-O-glucoside (10), kaempferol (11) and its 3-O-rhamnoside derivative (12), baicalein (13) and its 7-O-glucoside (14) and 7-O-glucuronide (15) derivatives, diosmetin (16) and its 7-O-rutinoside (17) derivative, hesperetin (19) and its 7-O-rutinoside (20) derivative and mangostin (18), a xanthonoid (Table S1) were assessed against HDF and B16F10 cancer cell lines to check their cytotoxicity at different concentration ranges from 0.1  $\mu$ M to 200  $\mu$ M. Almost all molecules inhibited cell growth in both cell lines in a dose dependent manner. At a higher concentration of molecules, *i.e.* at 100  $\mu$ M and 200  $\mu$ M, the cell viability was significantly low. However, the cell viability was comparable to the control in HDF cells at lower concentrations of 0.1  $\mu$ M, 1.0  $\mu$ M and 10  $\mu$ M. In comparison to aglycones, the glycosides were less toxic to both cell lines at higher dosages.

Among the quercetin derivatives, 5 was less toxic than the other glucose (3), rhamnose (2) and xylose (4) sugar conjugated at 3-OH position derivatives (Fig. 1). In comparison to rutinosides, 20 was more toxic than either 5 or 17. Compounds 5, 6, 9, 10, 11, 12, 13, 14, 15, 17 and 18 were non-toxic or showed significantly low toxicity to HDF cells at a maximum of up to 10  $\mu$ M concentration. However, relatively few molecules such as 2, 6, 13, 14 and 18 were non-toxic at a maximum of only 1  $\mu$ M concentration.

Among the three glucosides (3, 10, 14), 10 is less toxic than 14, followed by 3 in HDF cells while 14 is less toxic followed by 10 and 3 in the B16F10 cells. Similarly, among the rhamnosides (6, 8, 12), 12 was nontoxic in comparison to 6 and 8 in the HDF cells, while 6 was found to be non-toxic in the B16F10 cells. Compound 8 was comparatively more toxic than

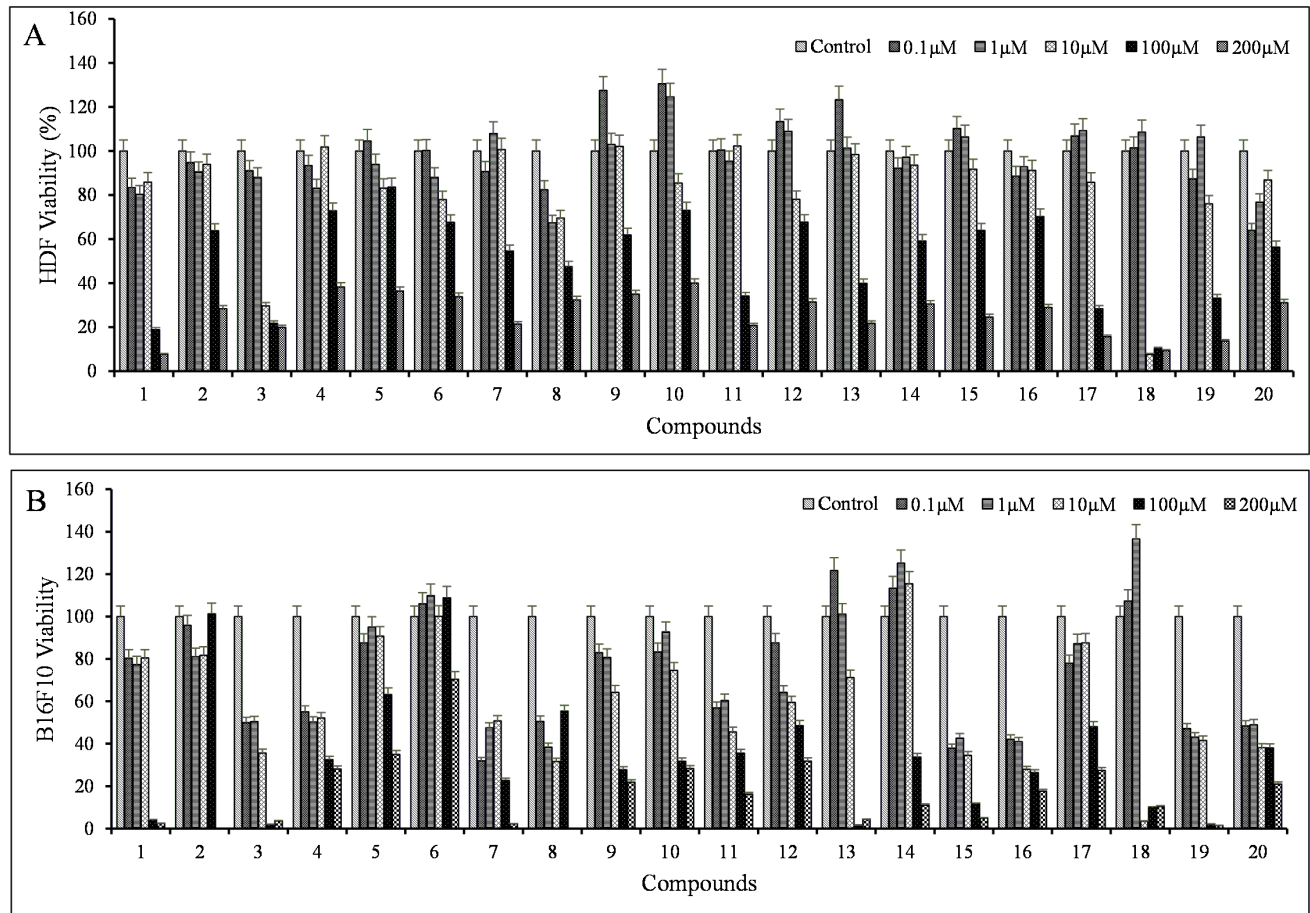


Fig. 1 — Cell viability assay of twenty different polyphenol and glycosides. (A) Human dermal fibroblast (HDF) cells; & (B) Skin cancer melanoma cells (B16F10)

the other rhamnosides in both cell lines. Similarly, among the aglycones (1, 7, 9, 11, 13, 16, 18 and 19), 1 was comparatively more toxic than the others in the HDF cells. Almost all aglycones were nontoxic at a maximum concentration of 1 μM. However, the scenario differed in the B16F10 cells, in which almost all aglycones exhibited toxicity except 13 and 18, even at a lower concentration of 0.1 μM.

Relatively non-toxic molecules (1, 2, 5, 6, 9, 10, 12, 13, 14, 17 and 18) were selected to study their potential activity for skin whitening. To study the effect of polyphenols on the inhibition of melanin biosynthesis, eleven different molecules were tested in the presence of 0.1 μM of compounds and the same amount of  $\alpha$ -MSH based on their toxicity data on B16F10 cells. Our results showed that, except molecules 6 and 13, all remaining nine compound treated cells had a lower content of melanin in comparison to the  $\alpha$ -MSH treated B16F10 cells. However, among these compound treated cells,

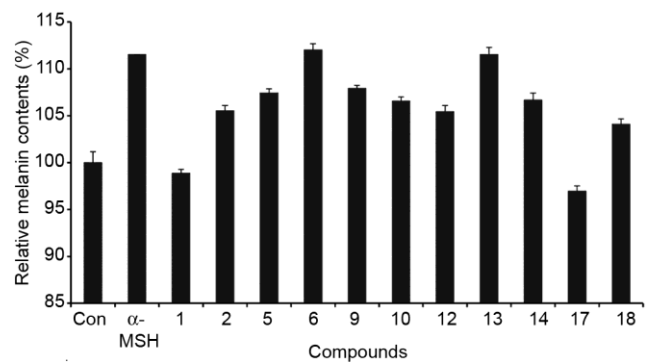


Fig. 2 — Relative melanin content analysis on B16F10 cells treated with selected eleven different compounds exhibiting lower cell cytotoxicity

only two molecules, 1 and 17 in the treated cells had less melanin than the control sample (Fig. 2). This result demonstrates that almost all nine molecules are capable of reducing the melanin content in the cells.

To study the effect on collagen biosynthesis, HDF cells treated with different sets of molecules at two

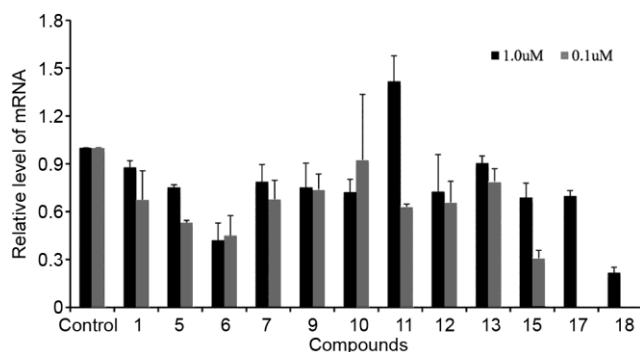


Fig. 3 — Transcript analysis of collagen type 1  $\alpha$  1 (*col1A1*) gene expression using qRT-PCR in twelve different compounds treated HDF cells. Low dose of compound 17 and 18 did not detect *Col1A1* mRNA

different concentrations (0.1  $\mu$ M and 1.0  $\mu$ M) were accessed for the activation of collagen type 1  $\alpha$  1 (*col1A1*) gene expression (Fig. 3). The expression level of the *col1A1* gene was checked using qRT-PCR with a set of primers as described in the experimental section. The results showed that compound 11 exhibited an enhanced level of gene expression when the cells were treated with 1.0  $\mu$ M of compound, at which concentration the molecule is completely non-toxic to cells. Other molecules such as the 1, 7, 10 and 13 treated HDF cells showed a comparable expression level, yet it was lower than that of the control sample. Compounds 10 and 13 were non-toxic to cells at a concentration of up to 0.1  $\mu$ M. However, compounds 1 & 7 were toxic, exhibiting approximately 10-20% reduction of cell viability. The compound 18 treated cells exhibited the lowest expression level among all compounds. Most of the biochemically synthesized and other glycosides of flavonoids are non-toxic to HDF cells. In some cases, the toxicity of molecules up to 10  $\mu$ M concentration was significantly low. Thus, although such molecules exhibited limited collagen biosynthesis activity on cells, they could be developed as new ingredients in different formulations of cosmetic products after extensive *in vivo* studies.

## Discussion

The study provided information on the possible modification of aglycone to enhance or alter certain functions of the aglycone molecule for cosmetic application. For example, no significant reduction is observed in melanogenesis upon modification of quercetin at the 3-OH position with various bulky hydrophilic sugar units such as glucose, rhamnose, xylose and rutinose. The modifications were made to

the 3-OH group of 1 and related molecule 6; the loss of potential was observed for these molecules to prevent melanocyte cells producing melanin. Similar activity was observed in 1 and modified compound 5 in the activation of collagen biosynthesis gene *col1A1* (Fig. 3). However, when the rutinose sugar was conjugated at the 7-OH position of another molecule, 16, generating rutinose 17, the melanin content in the cells was reduced. Similar activity was observed when the glucose unit was conjugated at the 7-OH position of 13 and 9. Moreover, we observed a similar result with apigenin and 7-OH modified glucoside derivative in collagen biosynthesis activation. In contrast, the conjugation of glucuronic acid at the same 7-OH position of baicalein reduced the *col1A1* gene expression level.

Although it is very difficult to generalize the exact modification required for the particular lead molecule to develop potential target activity, this result suggests the possible modification at the 7<sup>th</sup> OH position of flavonoids to reduce the melanin content or collagen biosynthesis enhancement in the cell. This ultimately facilitates skin whitening, preventing skin wrinkling and premature aging from the destructive effects of UV sun radiation and reactive oxygen species that induce melanocytes cells to produce melanin and the breakdown of collagen in skin collagenous tissues. Although several flavonoids and their glycosides are extracted from plants and studied for their potential applications as nutraceuticals<sup>33</sup>, pharmaceuticals<sup>35-37</sup>, cosmetics, human beneficial effects<sup>38</sup> and inhibitory effect against disease causing target proteins<sup>39</sup>, very few biochemically produced glycosides have been accessed for their potential application in the skin care products in cosmetics. These preliminary *in vitro* assays of naturally obtained flavonoids and biochemically synthesized glycoside derivatives provide information on the possible application of these molecules in skin care products.

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