# Flavonoids Enhance Melanogenesis in Human Melanoma Cells

Susumu TAKEKOSHI, Hidetaka NAGATA and Kanae KITATANI

*Department of Cell Biology, Division of Host Defense Mechanism, Tokai University School of Medicine*

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**Flavonoids are pigmentary compounds existing widely in plants. We have reported that quercetin (3, 3', 4', 5, 7-pentahydroxylflavone), one of the typical flavonoids, strongly promotes melanogenesis by melanocytes. Meanwhile, there are 8000 or more flavonoids having a chemical structure different from each other in the natural world. Their distinctive chemical properties suggest that they may be different in melanogenic actions. In the present study, the melanogenic actions of 14 flavonoids were analyzed to correlate their chemical structures with melanogenic actions. To evaluate the effects of flavonoids on melanogenesis, the HMV II cell line derived from human malignant melanoma was used. Flavonols including quercetin, kaempferol, rhamnetin and fisetin, flavones including apigenin, luteolin and chrysin, and isoflavones including genestein showed melanogenesis-promoting actions but rutin, robinetin, myricetin, ipriflavone, epigalocatechin gallate (EGCg) and naringin did not. From analyses of the relationships between the chemical structures of flavonoids and their melanogenesis-promoting actions, it was inferred that a hydroxyl group bound to the phenyl group plays an important role in stimulating melanogenesis. From the above results, 8 flavonoids were identified as melanogenesis promoters. Also, correlations were established between the melanogenesis-promoting actions of flavonoids and their chemical structures.**

**Key words: Flavonoid, tyrosinase, melanin, melanogenesis, HMVII cell**

## INTRODUCTION

Flavonoids are widely distributed in plants as pigmentary compounds, and are routinely consumed by humans from vegetables, fruits and grain. They have a variety of bioactivities, thus constituting one of the most important medically applicable natural substances [1–5]. Since the various bioactivities include anti-allergic and anti-viral actions, they are now attracting considerable attention as a potential medicinal drug [6, 7]. We have previously shown that quercetin, a representative flavonoid, is a powerful promoter of melanogenesis in the HMV-II cell line (Human Melanoma of Vagina II) [8–10]. Addition of quercetin to cell culture medium markedly increased melanin content, tyrosinase activity, and expression of tyrosinase protein. Tyrosinase activation by quercetin was blocked by actinomycin-D or cycloheximide suggesting that both transcriptional and translational events may have been involved in the stimulation of melanogenesis. These findings were also confirmed in a 3D dermal model and murine buccal follicular tissues.

Flavonoid is a generic name for a chemical compound possessing 2 phenyl groups (rings A and B) bound to one another with 3 carbon atoms (Fig. 1). Flavonoids are classified into 5 classes: flavonols, flavones, isoflavones, flavanols and flavones. Currently, around 8000 flavonoids have been identified. The side chains and locations of hydroxyl groups in the phenyl group may differ among the flavonoids, characterizing them with different chemical properties. It is therefore assumed that flavonoids with different chemical structures have different biological actions that include a stimulation of melanogenesis. The present study was aimed at determining the melanogenesis-promoting actions of 14 chemically different flavonoids to explore relationships between their chemical structures and melanogenesis-promoting actions.

## MATERIALS AND METHODS

### Chemicals and reagents

Mushroom tyrosinase, melanin, L-dopa, quercetin, rutin, kaempferol, robinetin, rhamnetin, fisetin, epigallocatechin gallate (EGCg), myricetin, chrysin, ipriflavone, genistein and naringin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Apigenin was purchased from Indofine (Somerville, NJ). Luteolin was purchased from Extrasynthese (Lyon, France). Ham's F12 medium and trypsin/EDTA were obtained from GIBCO (Carlsbad, CA, USA). Dimethylsulfoxide (DMSO) was purchased from Merck & Company (Darmstadt, Germany).

### Cell culture

HMVII is a human melanoma cell line that was established from a black-brown malignant melanoma in the vaginal wall of woman [11]. Human melanoma HMVII cells were kindly provided by the RIKEN Cell Bank. HMVII cells were cultured in Ham's F12 medium containing 10% fetal bovine serum (FBS) in a humidified 37°C atmosphere consisting of 5% $CO_2$ and 95% air. Cell plating densities were arranged so that cells were in the log phase of growth for the duration of incubation with drug. Subcultures of cells were plat-

Susumu TAKEKOSHI, Department of Cell Biology, Division of Host Defense Mechanism, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan Tel: +81-463-93-1121 ext. 2578 Fax: +81-463-94-2976 E-mail: takekos@is.icc.u-tokai.ac.jp

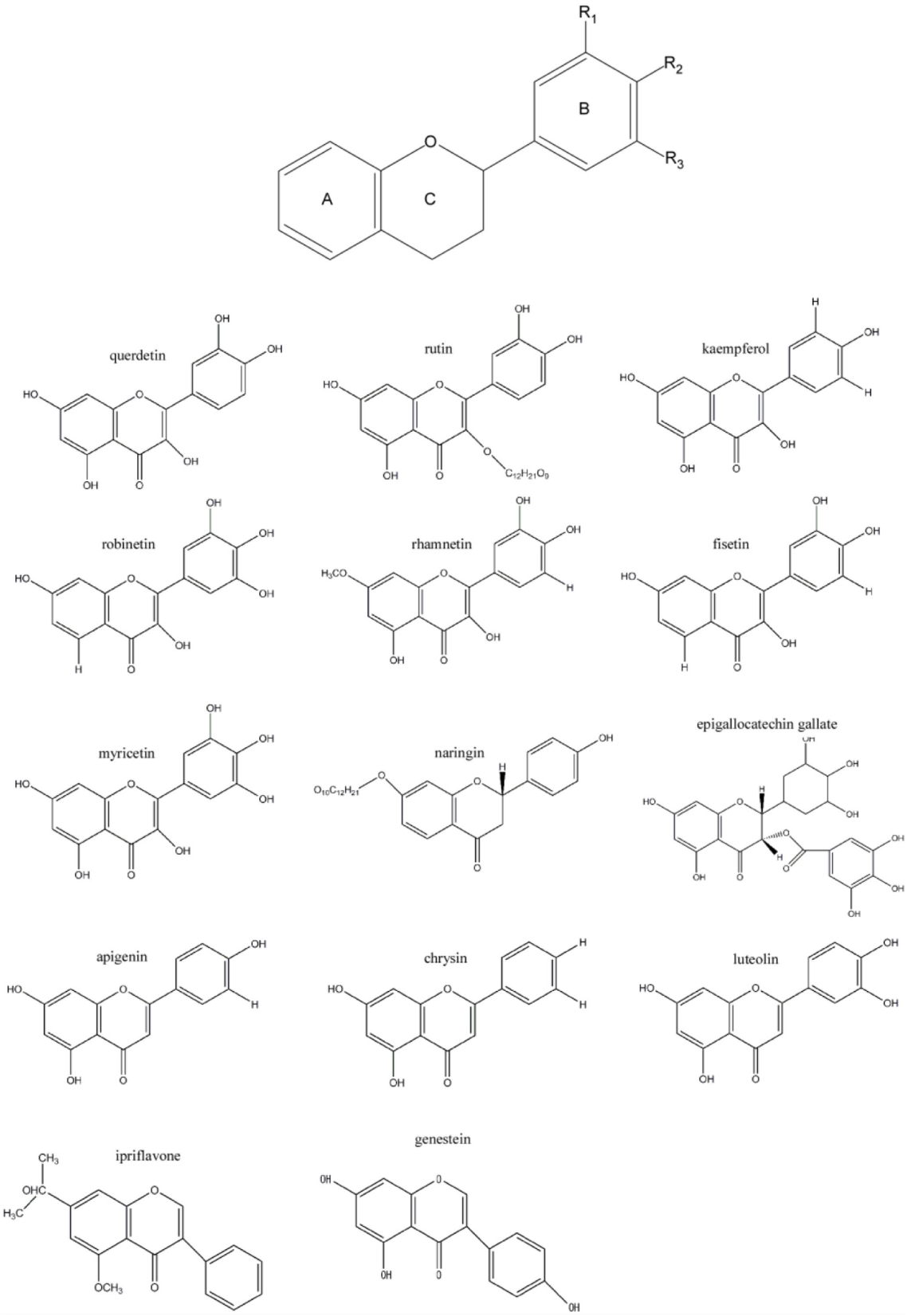


**Fig. 1** Structure of flavonoids

ed at a density of 4 × 104 cells/cm$^2$. Approximately 24 hr later, fresh medium and flavonoids were added, and cells were harvested 7 days after drug addition. For testing, flavonoids were dissolved in DMSO (dimethylsulfoxide). The final DMSO concentration was 0.1% in all experiments (including control) and had no measurable effect on HMVII cells.

**Measurement of melanin content**

The melanin content was determined as previ-

ously described [12]. After washes in PBS, cells were detached by short incubation in trypsin/EDTA (0.05%/0.02% in PBS). An aliquot was used for cell counts. The remaining cells were sonicated and incubated overnight in 500 μl 1 M NaOH. Melanin concentration was calculated by comparing the OD at 475 nm of unknown samples with a standard curve obtained with synthetic melanin.

**Tyrosinase activity**

Cellular tyrosinase activity using L-DOPA as the substrate was assayed by the method of Maeda and Fukuda [13]. $1 \times 10^6$ cells were washed with 10 mM phosphate-buffered saline (PBS) and lysed with 45 μl of 1% Triton X-100-PBS. After sonication, 5 μl of 20 mM L-DOPA were added to the wells. The 96 well plates were incubated at 37ºC for 1 hr, and the absorbance was measured at 475 nm in the model SPECTRAmax 250 microplate reader (Molecular Device Co). The absorbance values were compared with a standard curve obtained with purified mushroom tyrosinase. The standard curve was linear within the range of experimental values.

## RESULTS

**Effects of individual flavonoids on melanogenesis**

The effects on melanogenesis by 14 flavonoids differing from each other in chemical structure were tested in HMV II cells (Fig. 1). The 5 classes of flavonoids and compounds tested were 1) flavonol: quercetin, rutin, kaempferol, robinetin, rhamnetin, fisetin, and myrietin; 2) flavone: apigenin, luteolin, and chrysin; 3) isoflavone; ipriflavone, genestein, (4) flavanol: epigalocatechin gallate (EGCg); and 5) naringin: naringin. The concentration of the tested flavonoids was 20 μM DEMSO, which was a solvent for the tested flavonoids, was added to the control dish. Quercetin, kaempferol, rhamnetin and fisetin of the flavonol class exhibited a strong melanogenesis-promoting action but was not observed in rutin, robinetin, or myricetin. Rutin has a glycoside attached on ring A as well as 3 hydroxyl groups bound to ring B (fig. 1). Robinetin and myricetin have hydroxyl groups on R2 of ring B. Apigenin, luteolin, and chrysin of the flavone class and genestein of the isoflavone class significantly increased melanogenesis. However, ipriflavone, another isoflavone with a side chain bound to ring A lacked stimulatory activity. Such stimulatory action was also not shown by epigalocatechin gallate (EGCg) of the flavanol class or by naringin of the naringin class. Thus, it was determined that the chemical structure of flavonoids influences the stimulation of melanogenesis.

**Flavonoid effects on tyrosinase activity in HMVII cells**

Tyrosinase is a key enzyme in melanin synthesis by melanocytes. Using HMVII cells, the effects of 14 different flavonoids on tyrosinase activity were studied (Fig. 3). The concentration of each flavonoid in the medium was set at 20 μM. Quercetin, kaempferol, rhamnetin and fisetin of the flavonol class, when added to culture medium, resulted in a remarkable increase of tyrosinase activity of HMVII cells compared to the control medium containing only DEMSO. In contrast, rutin, robinetin or myricetin lacked a tyrosinase-stimulating action. These results were not inconsistent with melanogenesis-stimulating actions of flavonoids. Apigenin, luteolin and chrysin, which belong to the flavone class, significantly increased tyrosinase activity of HMVII cells. In the isoflavones, genestein showed tyrosinase-stimulation, but was not observed in ipriflavone, which has a side chain in ring A. Epigalocatechin gallate (EGCg) of the flavanol class and naringin of the naringin class did not show stimulatory activity. From the above results, it was clear that some flavonoids were able to stimulate the activity of tyrosinase, a rate-limiting enzyme of the melanin synthesis pathway in the cell. It was clarified that degrees of tyrosinase stimulation were positively correlated with amounts of melanin production and that promotion of melanogenesis by flavonoids depended on increased intracellular tyrosinase activity.

**Flavonoid concentration-dependent stimulation of melanogenesis**

Melanogenesis stimulation was exerted by quercetin, kaempferol, fisetin, rhamnetin, apigenin, luteolin, chrysin, or genestein. We had previously reported that quercetin had a concentration-dependent stimulatory action on melanogenesis [8]. Therefore, the 7 remaining flavonoids were examined for concentration-dependent production of melanin (Fig. 4). On treating HMVII cells with 1, 5, 10 and 20 μM flavonoids for 7 days, melanin content was significantly increased, as shown below: (results are expressed as multiples of the values for DMSO-treated control cells, at each of the flavonoid concentrations, respectively):

a) kaempferol to 1.21-, 1.44-, 1.54- and 3.30-fold;
b) apigenin to 1.20-, 1.75-, 2.08-, 5.53-fold;
c) luteolin to 1.49-, 2.76-, 4.64-, 8.90-fold;
d) chrysin to 1.35-, 1.89-, 2.27-, 4.17-fold;
e) fisetin to 1.11-, 1.36-, 1.39-, 2.94-fold;
f) rhamnetin to 0.84-, 0.87-, 1.43-, 3.50-fold;
g) genestein to 1.43-, 1.87-, 2.89-, 4.46-fold

As a result, concentration-dependent promotion of melanogenesis was demonstrated in all of the 7 flavonoids.

## DISCUSSION

It has now been clarified that 8 flavonoids, including quercetin, kaempferol, rhamnetin, fisetin, apigenin, luteolin, chrysin, and genestein, are promoters of melanin production in HMVII cells whose tyrosinase activity was stimulated by the flavonoids. Tyrosinase is a key enzyme for the melanin synthesis pathway of melanin-producing cells [14]. With these results, we established that these 8 flavonoids promoted melanin production via enhancing intracellular tyrosinase activity. We reported previously that quercetin, which was among the flavonoids in the present study, enhanced tyrosinase activity in HMVII cells and murine buccal follicular tissues through induction of tyrosinase protein expression [8–10]. It strongly suggested that the other 7 flavonoids might share the same mechanism of tyrosinase activity stimulation with quercetin.

Quercetin, kaempferol and rhamnetin, which are melanogenesis-stimulating members of the flavonol class, are characterized by the flavonoid structure where R2 of ring B binds a hydroxyl group (Fig. 1). It

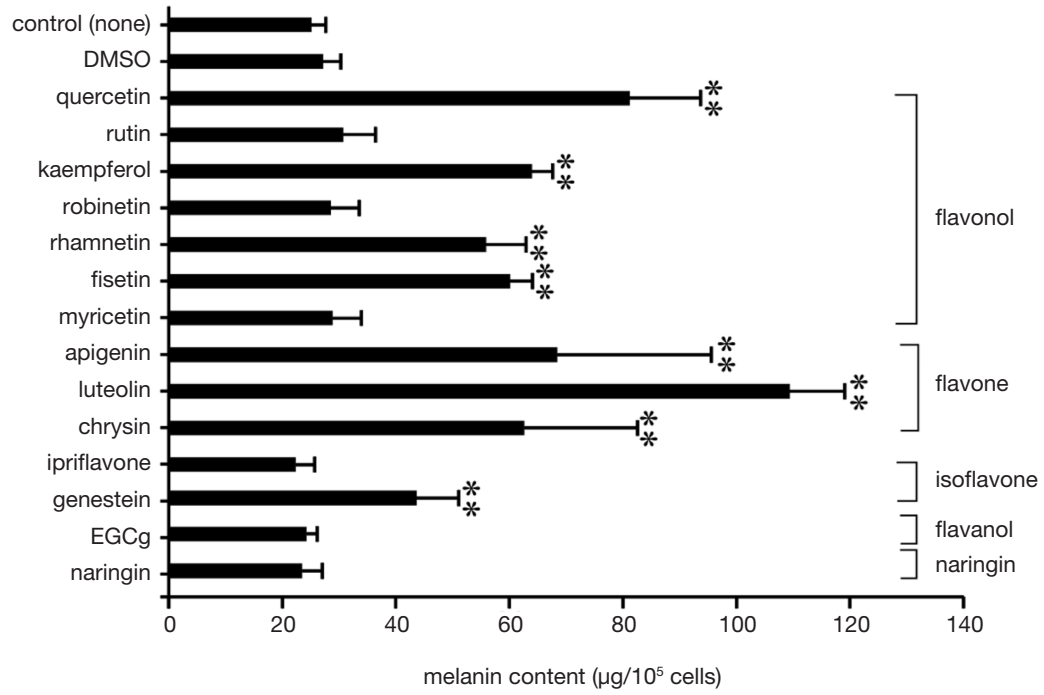


**Fig. 2** Flavonoids increased the melanin content in HMVII cells. Melanin content was determined by measuring the absorbance at 475 nm of HMVII cells treated with 20 μM flavonoids for 7 days, as described in Materials and Methods. Each value of melanin content is the mean ± SD of five determinations. Significant differences were determined by Student's t-test; $^{*}P < 0.01$, $^{**}P < 0.001$.

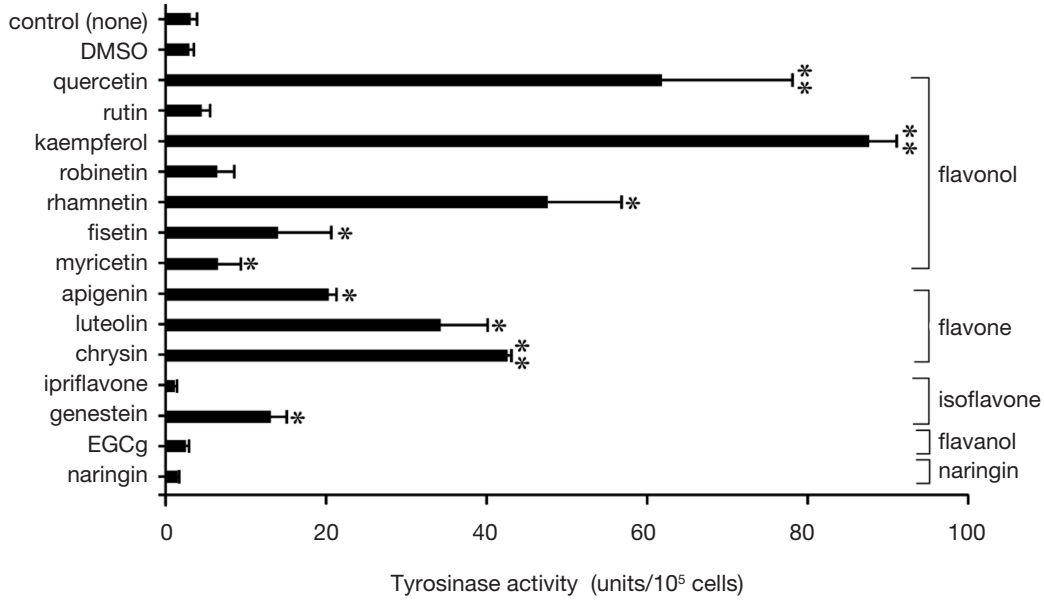


**Fig. 3** Flavonoids enhanced tyrosinase activity in HMVII cells. Tyrosinase activity was measured using L-DOPA (1 mM) as the substrate. Cells were treated with 20 μM flavonoids for 7 days. Tyrosinase activity is the mean ± SD of five determinations (n = 5). Significant differences were determined by Student's t-test; $^{*}P < 0.01$, $^{**}P < 0.001$.

is therefore inferred that a hydroxyl group at position R2 of ring B of the flavonoid skeleton is essential for the melanogenic action. Robinetin, myricetin and epigallocatechin gallate, although having a hydroxyl group at position R2 of ring B, did not show a melanogenesis-promoting action. These flavonoids are characterized by the presence of additional hydroxyl groups at positions R1 and R3 near R2 of ring B. Thus, the structure where hydroxyl groups are bound to R1, R2, and R3 of ring B may suppress a melanogenesis-stimulating action. Other flavonoids are not melanogenesis promoters, such as rutin and naringin which have a glycoside bound to ring A, and ipriflavone which has a similarly bound isopropoxy side chain. These side chains may suppress melanogenesis-stimulating actions.

Seven flavonoids from the present study have

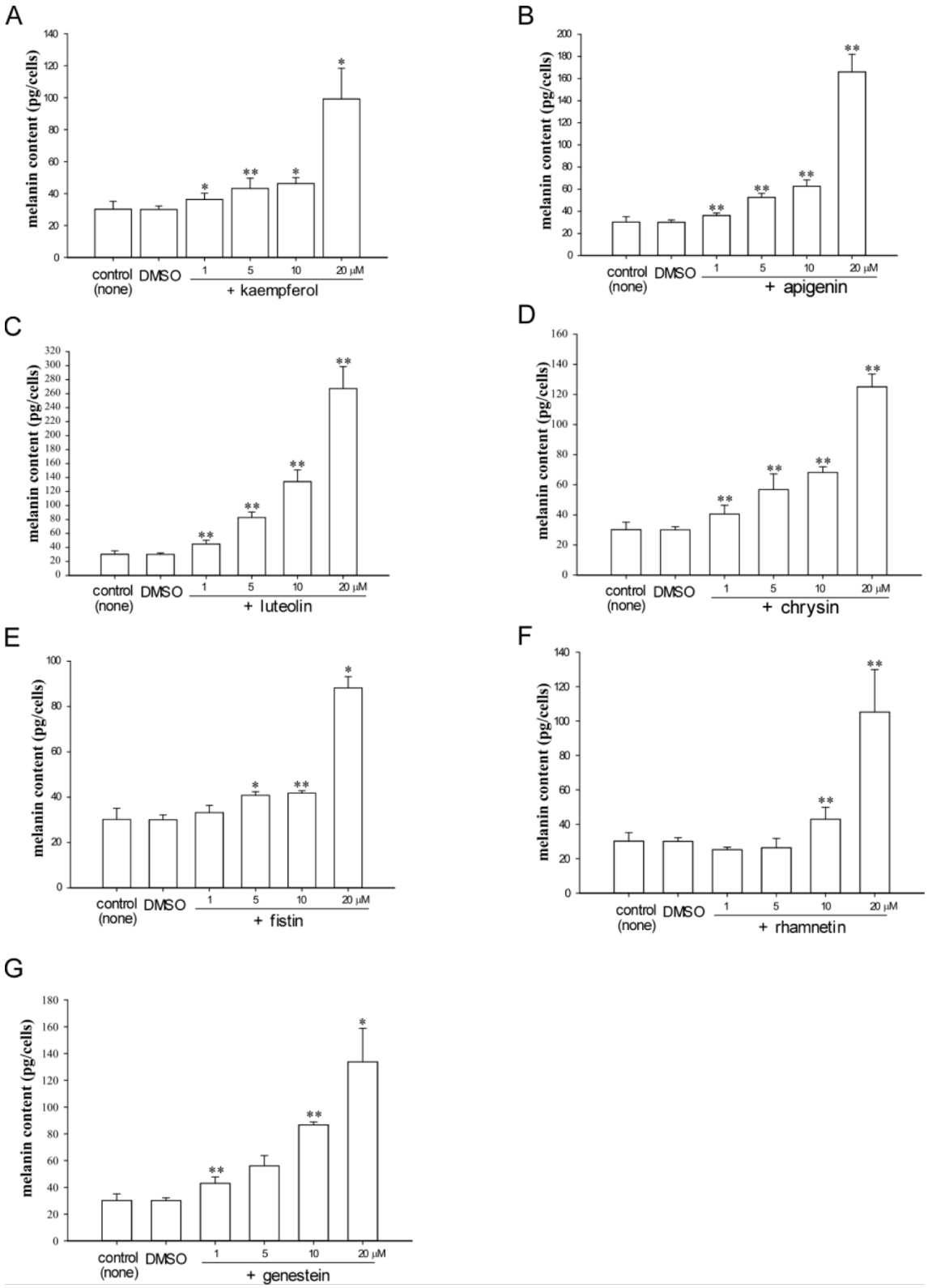


**Fig. 4** Various concentrations of flavonoids stimulated melanogenesis in dose dependent manner. Melanin content was determined by measuring the absorbance at 475 nm of HMVII cells treated with various concentrations (1, 5, 10, 20 μM). of flavonoids for 7 days, as described in Materials and Methods. Each value of melanin content is the mean ± SD of five determinations. Significant differences were determined by Student's t-test; $^{*}P < 0.01$, $^{**}P < 0.001$.

been added to the list of melanogenesis-promoting flavonoids that hitherto included only quercetin. It was also shown that flavonoid chemical structures have correlations with melanogenesis-stimulating actions. Flavonoids are expected to improve acquired depigmentation of skin and hair like vitiligo and white hair, respectively. Hopefully, the results of the present study will contribute to the development of more powerful

melanogenesis-stimulating agents.

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