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# 소사나무 잎 추출물 유래 멜라닌합성 저해 활성 성분

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# Anti-melanogenesis Active Constituents from the Extracts of *Carpinus turczaninowii* Leaves

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**요 약:** 소사나무(*Carpinus turczaninowii, C. turczaninowii*) 잎 추출물의 멜라닌 생성 억제활성을 B16F10 melanoma 세포를 이용하여 측정하였다. 그 결과, 에탄올 추출물(100 $\mu$g/mL)에서 72.2%의 생성 억제 효과를 확인하였으며, 동일 농도에서 MTT 세포독성은 거의 나타나지 않았다. 분획물(헥산, 에틸아세테이트, 부탄올 및 물)을 제조한 후 실험을 진행한 결과, 에틸아세테이트 분획에서 가장 우수한 활성이 관찰되었다. 에틸아세테이트 분획에서 활성성분을 규명하기 위하여 크로마토그라피를 진행하였으며, 4개의 화합물을 분리 동정하였다; ethyl gallate (1), quercetin rhamnose (2), kaempferol rhamnose (3), quercetin galloylrhamnose (4). 화합물의 구조규명은 핵자기공명분광기 등을 이용하여 이루어졌으며, 4개의 화합물 모두 소사나무 잎에서는 처음 분리된 물질이다. 분리된 화합물을 대상으로 멜라닌 생성 억제활성 실험을 진행한 결과, 화합물 4에서 세포독성 없이 농도의존적인 억제활성을 확인하였다. 또한, 화합물 4는 세포 내에서 티로시나제 발현양을 감소시키고 있음을 ELISA를 통하여 확인하였다. 이상의 결과를 바탕으로, 소사나무 잎 추출물이 화장품에서 미백제로 활용될 가능성이 있다고 판단된다.

**Abstract:** Melanin synthesis inhibition activities were investigated for the extracts prepared from the leaves of *Carpinus turczaninowii* (*C. turczaninowii*) by using B16F10 melanoma cells. As a result, the ethanol extract (100 $\mu$g/mL) showed 72.2% inhibition activities without cell toxicities in MTT assays. For the solvent fractions (*n*-hexane, ethyl acetate, *n*-butanol, water), the most potent activities were observed at the ethyl acetate fraction. To isolate the active constituents, the ethyl acetate fraction was further purified to afford four compounds; ethyl gallate (1), quercetin rhamnose (2), kaempferol rhamnose (3) and quercetin galloylrhamnose (4). The identification of the isolates was made by spectroscopic data including NMR spectra, and all of the compounds 1-4 were isolated for the first time from the leaves of *C. turczaninowii*. Anti-melanogenesis activities were studied for the isolates 1-4, and the compound 4 was determined to decrease the melanin synthesis dose-dependently without causing cell toxicities. ELISA measurement indicated that the isolate 4 decreased the contents of cell tyrosinase, a critical enzyme in melanogenesis. Based on these results, the extracts of *C. turczaninowii* were found to be applicable as whitening ingredients in cosmetic formulations.

***Keywords:*** *Carpinus turczaninowii, anti-melanogenesis, B16 melanoma cells, constituents*

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## 1. Introduction

Melanin is a polymeric pigment responsible for the red, brown and black colors in the human skin and hair. It is biosynthesized from the specialized group of cells known as the melanocytes localized in the epidermic layer of human skin. As the skin is exposed to external stimuli such as ultraviolet (UV) radiation, melanin synthesis is enhanced to protect the skin from harmful risk. However, the overproduction and accumulation of melanin in the epidermis could lead to skin pigment disorders including melasma, freckles and solar lentigo. Accordingly, the regulation of melanin production is an important strategy in the treatment of abnormal skin pigmentation for cosmetic and medicinal purposes[1-3].

In the process of melanogenesis, tyrosinase is the key enzyme catalyzing the first two steps of melanin production: the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and successive oxidation of the L-DOPA to L-dopaquinone. The resulting L-dopaquinone could be enzymatically transformed to 5,6-dihydroxyindole-2-carboxylic acid by tyrosinase-related protein-2 (TRP-2). There are two tyrosinase-related proteins, TRP-1 and TRP-2, which are structurally sharing 40-45% identity with tyrosinase[4].

A number of anti-melanogenic agents have been reported from both natural and synthetic sources, but only a few of them are used as skin-whitening agents primarily due to various safety concerns. Therefore, search of medicinal plants possessing high efficacy with low toxicity has been conducted for cosmetic and medicinal purposes[5].

Korean hornbeam *C. turczaninowii* is a deciduous woody plant belonging to the family *Betulaceae*. This flora is endemic to Korea, and can reach a height of 15 m. In the autumn, the fallen leaves of *C. turczaninowii* display a beautiful orange-red color and the tree is commonly used for bonsai in Korea. The wood is very hard, dense and fine textured, and has been used for making agricultural tools and furniture. Previous chemical investigation on this plant indicated only the existence of flavonoids such as naringenin and quercetin glycosides from the leaves[6]. We have recently reported the isolation of diarylheptanoids possessing anti-inflammatory activities from the ethanol extract of *C. turczaninowii*[7].

During the course of our study to investigate the bioactive compounds from the plants growing in Jeju Island, we observed that the *C. turczaninowii* leaves extracts have anti-melanogenesis activities on B16F10 melanoma cells. We decided to identify the active chemical constituents responsible for the activities. We herein report the isolation and identification of the constituents, and evaluation of their melanin synthesis inhibition activities.

## 2. Materials and Methods

### 2.1. Plant Materials

The leaves of *C. turczaninowii* were collected in May 2011 from Halla botanical garden in Jeju Island, Korea. Voucher specimen (sample number 308) was deposited at the herbarium of department of chemistry, Jeju National University.

### 2.2. Chemicals and Instruments

All solvents used were of analytical grade. $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were recorded on JNM-ECX 400 (FT-NMR system, JEOL, Japan) or AVANCE Ⅲ 500 (FT-NMR system, Bruker, Germany) instruments. Chemical shift ($\delta$) data were reported in ppm relative to the solvent used. Merck silica gel (0.063-0.2 mm) was used for normal phased column chromatography. Silica gel 60 $F_{254}$ coated on aluminum plates by Merck were used for thin layer chromatography (TLC). Gel filtration chromatography (GFC) was performed using Sephadex LH-20 (25-100 $\mu$m) from Fluka.

### 2.3. Extraction and Isolation of Compounds 1–4

Air-dried and powdered leaves of *C. turczaninowii* (700 g) were extracted three times with 70% ethanol using a magnetic stirrer at room temperature for 24 h. The extract was filtered and concentrated using a vacuum rotary evaporator at a maximum temperature of 40 ℃ to

afford a gummy extract (226 g). A portion of the extract (60 g) was suspended in water and partitioned to give *n*-hexane (Hex), ethyl acetate (EtOAc) and *n*-butanol (BuOH) soluble fractions.

The EtOAc-soluble fraction (5 g) was subjected to medium pressure liquid chromatography (MPLC) over $C_{18}$ silica gel with elution of $H_2O$-MeOH (10-100%) gradient to afford 44 fractions (fraction MP1-MP44). Among them, fraction MP10 was subjected to column chromatography (CC) over silica gel with $CHCl_3$/MeOH (3 : 1) to give compound 1 (51.7 mg). Compound 2 (87 mg) was obtained from fraction MP14 by recrystallization from methanol. Also, fraction MP16 was purified by CC over Sephadex LH-20 with $CHCl_3$/MeOH (6 : 1) to give the compound **2** (7.3 mg). Fraction MP28-32 were subjected to Sephadex LH-20 CC with $CHCl_3$/MeOH (3 : 1) to give compounds 3 (22.7 mg) and 4 (63.3 mg).

Ethyl gallate (1) $^1$H NMR (400 MHz, $CD_3OD$) $\delta_H$: 7.04 (1H, s, H-2, 6), 4.27 (2H, q, H-8), 1.34 (3H, t, H-9); $^{13}$C NMR (100 MHz, $CD_3OD$) $\delta_C$: 168.8 (C-7), 146.5 (C-3, 5), 139.7 (C-4), 121.8 (C-1), 110.0 (C-2, 6), 61.8 (C-8), 14.7 (C-9)

Quercetin rhamnose (2) $^1$H NMR (400 MHz, $CD_3OD$) $\delta_H$: 7.33 (1H, d, $J$ = 1.8 Hz, H-2'), 7.32 (1H, dd, $J$ = 1.8, 8.2 Hz, H-6'), 6.91 (1H, d, $J$ = 8.2 Hz, H-5'), 6.37 (1H, d, $J$ = 1.8 Hz, H-8), 6.20 (1H, d, $J$ = 1.8 Hz, H-6), 5.34 (1H, d, $J$ = 1.4 Hz, H-1''), 4.22-3.33 (4H, H-2''-5''), 0.94 (3H, d, $J$ = 5.9 Hz, H-6''); $^{13}$C NMR (100 MHz, $CD_3OD$) $\delta_C$: 179.8 (C-4), 166.0 (C-7), 163.4 (C-5), 159.5 (C-9), 158.7 (C-2), 150.6 (C-4'), 146.6 (C-3'), 136.4 (C-3), 123.1 (C-1'), 123.0 (C-6'), 117.1 (C-2'), 116.5 (C-5'), 106.0 (C-10), 103.7 (C-1''), 100.0 (C-6), 94.8 (C-8), 73.4 (C-4''), 72.2 (C-3''), 72.2 (C-5''), 72.0 (C-2''), 17.8 (C-6'')

Kaempferol rhamnose (3) $^1$H NMR (500 MHz, $CD_3OD$) $\delta_H$: 7.76 (2H, d, $J$ = 7.0 Hz, H-2', 6'), 6.92 (2H, d, $J$ = 7.0 Hz, H-3', 5'), 6.36 (1H, d, $J$ = 2.0 Hz, H-8), 6.18 (1H, d, $J$ = 2.0 Hz, H-6), 5.37 (1H, d, $J$ = 1.5 Hz, H-1''), 4.22-3.71 (4H, H-2''-5''), 0.92 (3H, d, $J$ = 6.0 Hz, H-6''); $^{13}$C NMR (125 MHz, $CD_3OD$) $\delta_C$: 179.7 (C-4), 166.0 (C-7), 163.3 (C-5), 161.7 (C-4'), 159.4 (C-9), 158.7 (C-2), 136.3 (C-3), 132.0 (C-2', 6'), 122.8 (C-1'), 116.6 (C-3', 5'), 106.0 (C-10), 103.6 (C-1''), 99.9 (C-6), 94.9 (C-8), 73.3 (C-4''), 72.2 (C-3''), 72.2 (C-5''), 72.1 (C-2''), 17.8 (C-6'')

Quercetin galloylrhamnose (4) $^1$H NMR (400 MHz, $CD_3OD$) $\delta_H$: 7.36 (1H, d, $J$ = 2.0 Hz, H-2'), 7.34 (1H, dd, $J$ = 2.0, 8.5 Hz, H-6'), 7.07 (2H, s, H-2''', 6'''), 6.93 (1H, d, $J$ = 8.5 Hz, H-5'), 6.36 (1H, d, $J$ = 2.0 Hz, H-8), 6.18 (1H, d, $J$ = 2.0 Hz, H-6), 5.64 (1H, dd, $J$ = 1.5, 2.0 Hz, H-2''), 5.50 (1H, d, $J$ = 1.5 Hz, H-1''), 4.03-3.46 (3H, H-3''-5''), 1.03 (3H, d, $J$ = 5.5 Hz, H-6''); $^{13}$C NMR (100 MHz, $CD_3OD$) $\delta_C$: 179.4 (C-4), 167.5 (C-7'''), 165.9 (C-7), 163.2 (C-5), 159.3 (C-9), 158.5 (C-2), 149.9 (C-4'), 146.6 (C-3''', 5'''), 146.5 (C-3'), 140.0 (C-4'''), 135.7 (C-3), 122.9 (C-6'), 121.3 (C-1'), 121.3 (C-1'''), 117.0 (C-5'), 116.5 (C-2'), 110.4 (C-2''', 6'''), 105.9 (C-10), 100.6 (C-1''), 99.9 (C-6), 94.8 (C-8), 73.8 (C-3''), 73.6 (C-2''), 72.3 (C-5''), 70.2 (C-4''), 17.9 (C-6'')

### 2.4. Cell Culture

B16F10 murine melanoma cell was purchased from the American Type Cell Culutre (ATCC, USA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Inc., USA) supplemented with 10% fetal bovine serum (FBS, Gibco Inc., USA) and 1% penicillin/streptomycin at 37 ℃ in a humidified 95% air/ 5% $CO_2$ atmosphere.

### 2.5. Determination of Cellular Tyrosinase Activity/Melanin Contents

B16F10 murine melanoma cells were seeded into 6-well cell culture plates at a density of $1.0 \times 10^5$ cells/well. A day later, the cells were stimulated with $\alpha$-melanocyte stimulating hormone ($\alpha$-MSH) (100 nM) and treated with samples. The resulting cells were incubated at 37 ℃, 5% $CO_2$ condition for three days. The cells were washed with 1X phosphate buffered saline (PBS) and then collected using 1X trypsin-ethylenediaminetetraacetic acid (EDTA), after which they were lysed with 0.2 mM phenylmethylsulfonyl fluoride (PMSF)

and 1% triton-X 100 in 67 mM sodium phosphate buffer (pH 6.8). The samples were sonicated and centrifuged at 15,000 rpm for 20 min at 4 ℃. Supernatant (80 $\mu$L) was placed in a 96-well plate and mixed with 25 mM L-DOPA (40 $\mu$L) and 67 mM sodium phosphate buffer (120 $\mu$L, pH 6.8). After reacting at room temperature for at least 1 h, the absorbance was measured at 475 nm. To dissolve the melanin, 1 N NaOH was added to the pellets and subsequently incubated at 70 ℃ for 4 h. Absorbance of total protein was measured using the supernatant mixture and Bradford reagent.

### 2.6. Cell Viability Assay

The cell viability was determined by 3-(4,5-Dimethylthiazol -2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability assay. Briefly, cells were seeded into 6-well cell culture plates. After 24 h, the cells were stimulated with $\alpha$-MSH (100 nM) and treated with samples, and incubated for 72 h at 37 ℃ under 5% $CO_2$ condition. MTT reagent (5 mg/mL in PBS) was added to each well in a 1/10 volume of medium. Cells were incubated at 37 ℃ under 5% $CO_2$ condition for 3 h. Finally, the media was removed, and the formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm. Cell viability was evaluated as the relative absorbance of the control group.

### 2.7. Statistical Analysis

The student's *t*-test and one-way ANOVA were used to statistically significant differences between the values for the various experimental and control groups. Data are expressed as means ± standard errors (S.E.M.) and the results represent at least three independent experiments. Values are the mean ± S.E.M. of triplicate experiments ($^{*}p < 0.05$, $^{**}p < 0.01$).

## 3. Results and Discussion

The extract was prepared with 70% aqueous ethanol. The inhibitory effects on melanin production for the extract were measured by using B16F10 melanoma cells. When the cells were treated with $\alpha$-MSH, their melanin

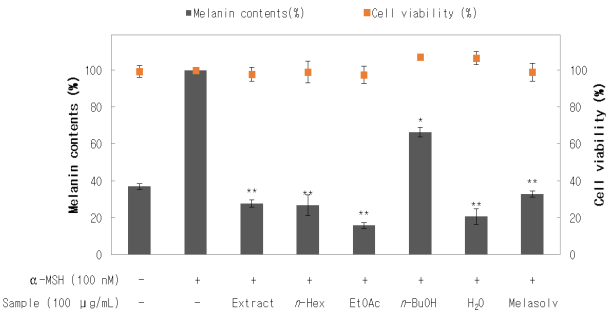


**Figure 1.** Effects of extract and solvent fractions from *C. turczaninowii* on melanin contents in $\alpha$-MSH-stimulated B16F10 melanoma cells. The cells were stimulated with 100 nM of $\alpha$-MSH only, or with $\alpha$-MSH plus *C. turczaninowii* extract and solvent fractions (100 $\mu$g/mL, melasolv; 20 $\mu$M) for 72 h. Melanin contents were determined by the absorbance at 405 nm. The data represent the mean ± S.D. of triplicate experiments. Values are the mean ± S.E.M. of triplicate experiments ($^{*}p < 0.05$, $^{**}p < 0.01$).

biosynthesis was activated. In this test, as shown in Figure 1, treatment of the ethanol extract (100 $\mu$g/mL) to the activated cells dropped the melanin production by 72.2%. A synthetic compound melasolv being applied as a whitening agent in cosmetic formula was used as a positive control.

The extract was fractionated into *n*-Hex, EtOAc, *n*-BuOH and water-soluble portions. Each fraction was also subjected to the activity test for melanin biosynthesis. In this test, EtOAc fraction exhibited the most potent activity with 84.3% inhibition of melanin production at 100 $\mu$g/mL concentration. The cell toxicities were examined for the ethanol extract and solvent fractions by using MTT test. As shown in Figure 1, cell toxicities were not detected as cell viabilities were maintained at the indicated concentrations for all of the fractions.

It is of significance to identify the second metabolite responsible for the biological activity in the extract. As EtOAc fraction possess the highest activity without causing cell toxicities, it was selected and subjected for further purification operations. Repeated chromatographic separation procedures led to isolation of four compounds, which were identified as ethyl gallate (1), quercetin rhamnose (2), kaempferol rhamnose (3) and quercetin galloylrham-

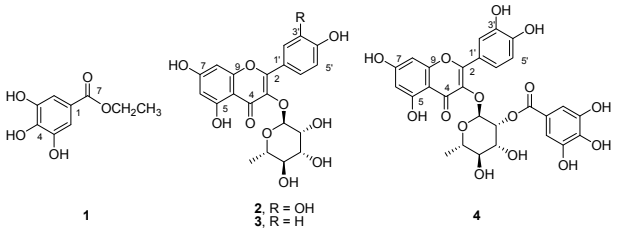


**Figure 2.** Chemical structures of isolated compounds 1-4 from *C. turczaninowii* leaves.

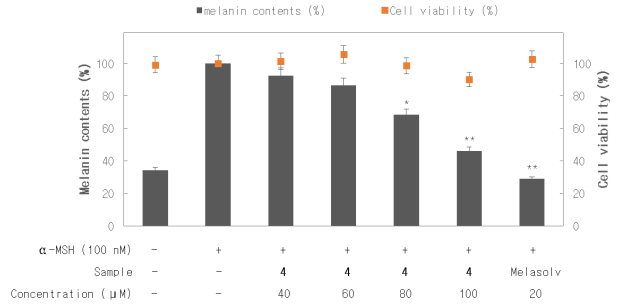


**Figure 3.** Effects of isolated compound 4 from *C. turczaninowii* on melanin contents in $\alpha$-MSH-stimulated B16F10 melanoma cells. The cells were stimulated with 100 nM of $\alpha$-MSH only, or with $\alpha$-MSH plus isolated compound 4 for 72 h. Melanin contents were determined by the absorbance at 405 nm. The data represent the mean ± S.D. of triplicate experiments. Values are the mean ± S.E.M. of triplicate experiments ($^{*}p < 0.05$, $^{**}p < 0.01$).

nose (4) (Figure 2). The chemical structures of the compounds 1-4 were determined by the examination of their spectroscopic data including 1D and 2D NMR spectra as well as comparison of their data to the literature values[8-11]. Even though the compounds 1-4 were not new compounds, they were isolated for the first time from the extract of *C. turczaninowii*.

When the isolated compounds 1-4 were tested for the melanin synthesis inhibition, the galloyl flavonoid 4 exhibited strong activities with 53.3% inhibition at 100 $\mu$M concentrations without causing cell toxicities (Figure 3).

Subsequently, anti-melanogenesis activity and cell-toxicities were examined with varying substrate concentrations (40-100 $\mu$M). As shown in Figure 3, the galloyl flavonoid 4 was shown to inhibit the melanin synthesis with dose-dependent manner. At the concentration up to

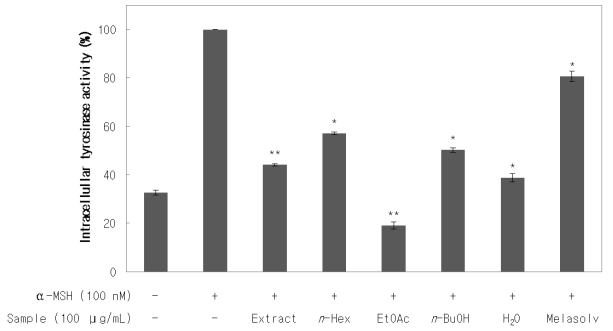


**Figure 4.** Effects of extract and solvent fractions from *C. turczaninowii* on intracellular tyrosinase activities in $\alpha$-MSH-stimulated B16F10 melanoma cells. The cells were stimulated with 100 nM of $\alpha$-MSH only, or with $\alpha$-MSH plus *C. turczaninowii* extract and solvent fractions (100 $\mu$g/mL, melasolv; 20 $\mu$M) for 72 h. Absorbance was measured at 475 nm with a ELISA. The data represent the mean ± S.D. of triplicate experiments. Values are the mean ± S.E.M. of triplicate experiments ($^{*}p < 0.05$, $^{**}p < 0.01$).

100 $\mu$M, cell viabilities were maintained over 90% indicating 4 has very low cytotoxicities. On the other hand, the ethyl gallate (1) showed a moderate activity showing decrease of melanogenesis by 13.9% at 100 $\mu$M concentration without cytotoxcities. At the higher concentration (200 $\mu$M), however, the compound 1 induced considerable cytotoxicities (64.4% cell viabilities) with lowering melanogenesis by 31.3%. In addition, the othe isolates 2 and 3 showed no activities at the same concentration of 250 $\mu$M (data not shown).

In order to determine the mechanism of anti-melanogenesis, tyrosinase contents produced inside the B16F10 melanoma cells were determined by ELISA. Tyrosinase is a metalloenzyme playing the key role to synthesize melanin from L-tyrosine in the skin melanocyte. L-tyrosine is oxidized to L-DOPA and subsequently to dopaquinone by tyrosinase, which is the rate-determining step in melanin biosynthesis. When the melanoma cell is activated by $\alpha$-MSH, the induction of tyrosinase is increased resulting in melanin synthesis activation.

As the extract (100 $\mu$g/mL) was added, the production of the tyrosinase enzyme was decreased by 55.9% (Figure 4). The solvent fractions were also subjected to the tests by ELISA, EtOAc fraction showed the most potent activ-

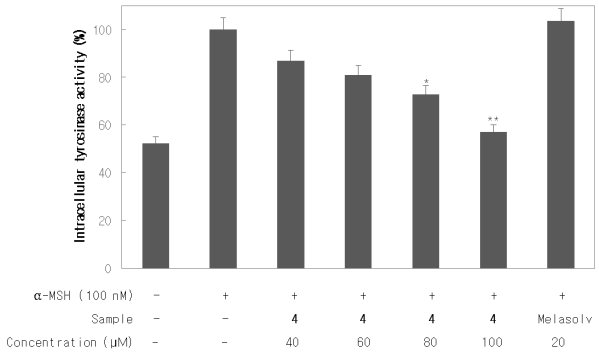


**Figure 5.** Effects of isolated compound 4 from *C. turczaninowii* on intracellular tyrosinase activities in $\alpha$-MSH-stimulated B16F10 melanoma cells. The cells were stimulated with 100 nM of $\alpha$-MSH only, or with $\alpha$-MSH plus isolated compound 4 for 72 h. Absorbance was measured at 475 nm with a ELISA. The data represent the mean ± S.D. of triplicate experiments. Values are the mean ± S.E.M. of triplicate experiments ($^{*}p < 0.05$; $^{**}p < 0.01$).

ity on tyrosinase production with 81.0% inhibition at 100 $\mu$g/mL. Melasolv (19.3% inhibition at 20 $\mu$M) was used as the positive control. When the isolated compound 4 was examined with varying concentrations, it also showed inhibitory activities for the tyrosinase enzyme production in a dose-dependent manner (Figure 5). These data demonstrated that the melanin synthesis inhibition by the *C. turczaninowii* extract could be associated with the down-regulation of the tyrosinase enzyme in the melanoma cells.

## 4. Conclusion

The ethanol extract (100 $\mu$g/mL) of *C. turczaninowii* exhibited potent anti-melanogenesis activities on B16F10 melanoma cells without cell toxicities. Four solvent fractions *n*-Hex, EtOAc, *n*-BuOH, water were prepared, and the ethyl acetate fraction possessed the most potent activities (84.3% inhibition at 100 $\mu$g/mL). Phytochemical investigation of the ethyl acetate fraction led to isolate four compounds; ethyl gallate (1), quercetin rhamnose (2), kaempferol rhamnose (3) and quercetin galloylrhamnose (4). All of the compounds 1-4 were isolated for the first time from the leaves of *C. turczaninowii*. The galloyl flavonoid 4 was determined to decrease the melanin synthesis in dose dependent manner in B16F10 melanoma cells. In addition, the compound 4 decreased the contents of cell tyrosinase, a critical enzyme in melanogenesis, determined by ELISA analysis. In this study, the extracts of *C. turczaninowii* containing the quercetin galloylrhamnose (4) were found to be applicable as anti-melanogenesis ingredients in cosmetic formulations.

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