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Synthesis of Quercetin and Luteolin Derivatives with Cell Proliferation Inhibitory Activity and Toxicity in B16 Melanoma Cells

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ABSTRACT. Melanoma is a malignant skin tumor caused by damage to melanocytes that can spread to other organs. Hence, various studies have been conducted on preventing the spread of melanoma. Flavonoid-structured substances such as apigenin and galanzin are effective therapeutic agents for inhibiting the proliferation and metastasis of melanoma. In this study, luteolin, quercetin, and their respective derivatives were synthesized. These compounds inhibited cell proliferation of B16 melanoma cells. These results confirmed that the derivatives of quercetin and luteolin may be useful as therapeutic agents to prevent melanoma metastasis.

Key words: Quercetin, Luteolin, Melanoma

INTRODUCTION

Melanoma, caused by UV-damaged melanocytes that produce pigments on the surface of the skin, is a malignant skin tumors accounting for 75% of skin cancer mortality.[1-3] In addition, melanoma easily metastasizes to the lungs, lymph nodes, and bones.[4,5] Thus, several studies have been conducted to determine substances that prevent the spread of melanoma. Rho kinase inhibitors, such as AT13148 and CCT129254 (*Fig*. 1), block melanoma cell migration and inhibit metastasis.[6] Galangin (*Fig*. 1) inhibits the proliferation and metastasis of B16 melanoma cells by inhibiting the focal adhesion kinase (FAK) of tyrosine kinase, which is involved in the proliferation, adhesion, and invasion of cancer cells.[7]

Melanoma biosynthesizes melanin, a pigment found in human hair and skin that plays an important role in protecting the skin from UV damage.[8-11] The decreased rate of melanin production is associated with aging and stress and causes gray hair and leukopenia. However, the overproduction of melanin because of sunlight can stain the skin or make it darker. Many foods and herbs have been reported to have an effect in modulating melanin production to maintain the health and appearance of the human body.[12-18]

Quercetin (*Fig.* 2) is a flavonol-structured compound found in many fruits, vegetables, grains, and leaves, including capers, red onions, cranberries, kale, and radish leaves.[19] It has antioxidant effects to suppress endothelial cell death by oxidation.[20] In addition, it is being developed as a treatment for various diseases, such as the treatment of colorectal cancer through inhibition of the enhancer of zeste homologue 2 (EZH2),[21] the induction of cell cycle arrest and apoptosis of breast cancer cells,[22] and the treatment of type 2 diabetes through the formation of fatty acids and hybrid molecules.[23,24]

Luteolin (*Fig.* 2) is a flavonol-structured compound found in many herbal extracts, including celery, bell peppers, parsley, perilla leaves, seeds, and chamomile, and has shown anti-inflammatory effects in *in vivo* and *in vitro*

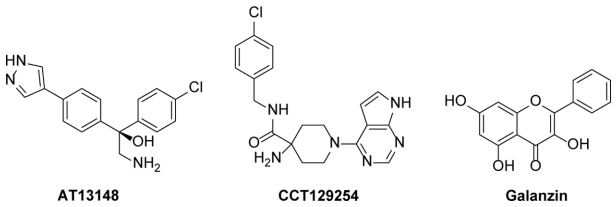


***Figure* 1.** Melanoma metastasis inhibitor.

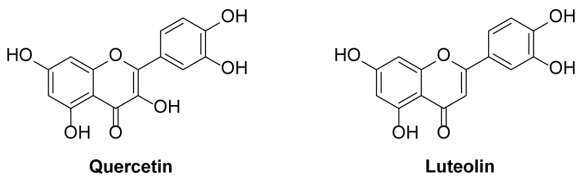


***Figure* 2.** Structure of quercetin and luteolin.

Jongyun Jang and Seong Uk Lee contributed equally to this article.

experiments.[25,26] It also has anticancer effects, such as inhibition of melanin production and proliferation in B16 melanoma cells,[27] inhibition of insulin-like growth factor 1 receptor (IGF-1R) involved in prostate cancer growth,[28] and cell cycle arrest induced in lung cancer cells and apoptosis.[29]

CHEMISTRY

Luteolin was synthesized using the methods described by Wang *et al*. and Zhang *et al*.[30,31] Compound **3** was synthesized via Friedel-Crafts acylation of phloroglucinol **1** and dimethylation of compound **2** using dimethyl sulfate as methylating agent. Aldol condensation using compound **3** and veratraldehyde synthesized chalcone **4**. Compound **5** was synthesized by cyclization of compound **4** using iodine and DMSO. Demethylation of 3′-, 4′-, 5- and 7-methoxy groups using pyridine HCl in H_2O at 160 ℃ produced compound **6**. As there is no significant difference in the reactivity between 7-hydroxyl and 4′-hydroxyl groups in luteolin **6**, the 3′- and 4′-hydroxyl groups were protected using dichlorodiphenylmethane to give the corresponding acetal **7**.[32] Alkylation was carried out using an appropriate alkyl iodide. Then, hydrolysis of the acetal structures of 3′ and 4′-hydroxyl groups using AcOH/H_2O yielded compounds **10** and **11**.

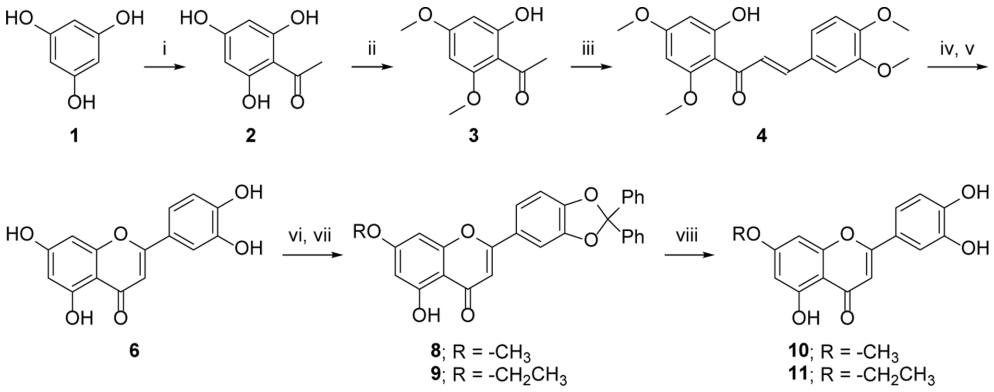
Reagents and conditions; (i) Ac_2O, $BF_3{\cdot}Et_2O$, EtOAc, r.t., 16h, 89%; (ii) dimethyl sulfate, K_2CO_3, acetone, 60 ℃, 21h, 85%; (iii) veratraldehyde, KOH, MeOH, 90 ℃, 24h, 63%; (iv) iodine, DMSO, 100 ℃, 12h, 96%; (v) pyridine HCl, H_2O, 160 ℃, 14h, 84%; (vi) Ph_2CCl_2, Ph_2O, 180 ℃, 30 min, 85%; (vii) alkyl iodide, K_2CO_3, DMF, r.t., 12h, 81%; (viii) AcOH/H_2O(80/20), 120 ℃, 6h.

The acylation of the 7-hydroxyl group occurred in compound **7** owing to the reactivity of the luteolin hydroxyl groups. Thereafter, compounds **16-19** in which the acetal group was deprotected, were obtained via hydrolysis.

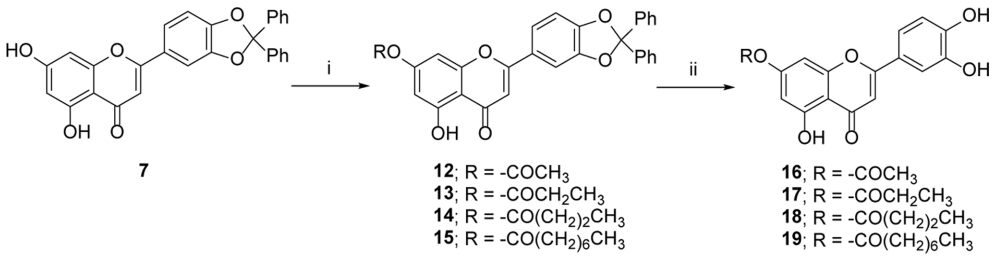
Reagents and conditions; (i) R_2O, pyridine, 80 ℃, 3h, 65~74%; (ii) 10% Pd/C, H_2, THF/EtOH(1/1), r.t., 12-24h, 30~68%.

The alkylation reaction of the 7-hydroxyl group in quercetin proceeded in a similar manner to that observed in luteolin. To selectively activate only the 7-hydroxyl groups, the 4′-hydroxyl groups, which are more reactive than the 7-hydroxyl groups, were protected with an acetal structure using dichlorodiphenylmethane. The protection of the 3-hydroxyl group in **20** proceeded via benzylation. The alkylation reaction was performed using an alkyl iodide. Debenzylation of compounds **27-31** was carried out by hydrogenation, followed by hydrolysis of the acetal structure of 3′, 4′-hydroxyl groups using AcOH/H_2O.

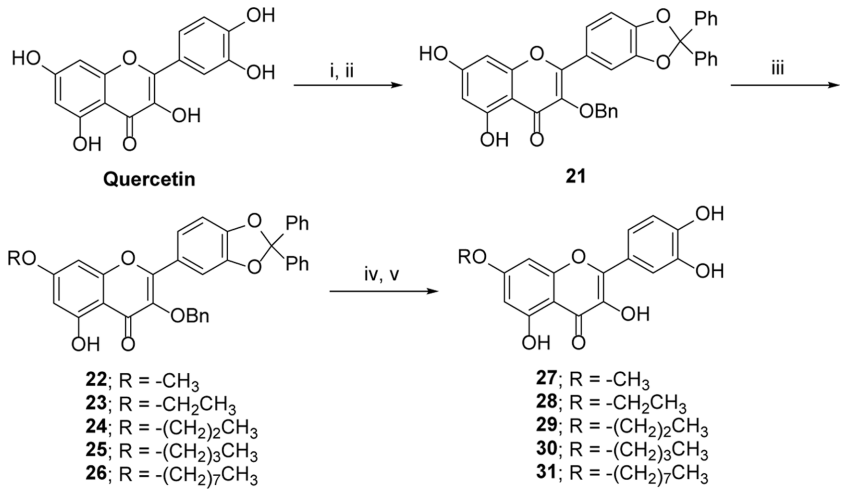
Reagents and conditions; (i) Ph_2CCl_2, Ph_2O, 180 ℃,



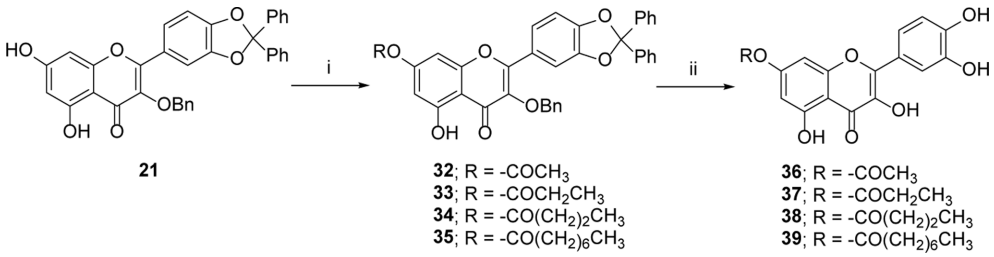
***Scheme* 1.** Synthesis of 7-O-alkylated luteolin.



***Scheme* 2.** Synthesis of 7-O-acylated luteolin.



***Scheme* 3.** Synthesis of 7-O-alkylated quercetin.



***Scheme* 4.** Synthesis of 7-O-acylated quercetin.

30 min, 63%; (ii) BnBr, K_2CO_3, DMF, r.t., 12h, 55%; (iii) alkyl iodide, K_2CO_3, DMF, r.t., 12h, 53~79%; (iv) 10% Pd/C, H_2, r.t., 12h~48h; (v) AcOH/H_2O, 120 ℃, 6h, 64~76%.

The acylation reaction of the 7-hydroxyl group in quercetin proceeded in a similar manner to that observed in luteolin. Thereafter, compounds **36-39** in which the benzyl and acetal groups were deprotected, were obtained through hydrogenation.

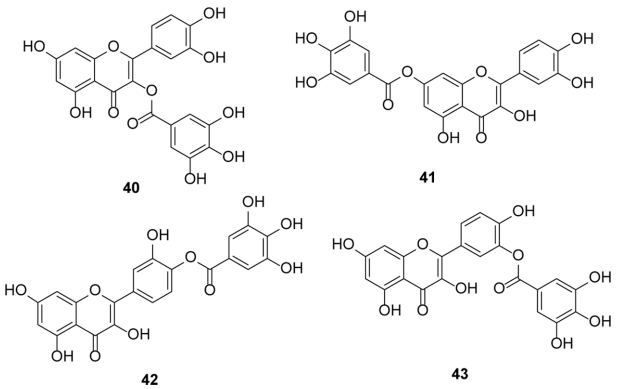


***Figure* 3.** Structure of mono-gallic acid coupling compounds of quercetin.

Reagents and conditions; (i) acid anhydride, pyridine, 80 ℃, 3h, 31~76%; (ii) 10% Pd/C, H_2, THF/EtOH (1:1), r.t., 12-24h, 35~44%.

Mono-gallic acid coupling compounds of quercetin (*Fig.* 3) were synthesized using a method previously reported. Compounds **40**, **41**, and **42** were synthesized as described in our previous study.[33] Compound **43** was prepared by Thapa *et al*. as presented in this study.[24]

EXPERIMENTAL

General Materials and Methods

Reagents and solvents were commercially available and used without purification. Reactions were monitored by thin-layer chromatography carried out on 0.25 mm Merck silica gel plates (60F254) using UV light as the 254 nm visualization agent. The separation of samples by flash chromatography was performed using Merck silica gel 60 (40-63 μm). ^{1}H NMR spectra were obtained using a JEOL superconducting magnet JMTC-400/54/JJ/YH (400 MHz). Chemical shifts were recorded in ppm downfield from tetramethylsilane (TMS), and coupling constant (*J*) values are given in Hertz.

Experimental Procedure

1-(2,4,6-Trihydroxy-phenyl)ethanone (2)

Acetic anhydride (7.5 mL, 79.3 mmol) was added to a solution of phoroglucinol (10.00 g, 79.3 mmol) in EtOAc (200 mL). BF_3OEt_2 (11.8 mL, 95.2 mmol) in EtOAc (50 mL) were dropwised in the reaction vessel for 1h and the mixture was stirred at 70 ℃ for 10h. The resulting mixture was added water (300 mL) and extracted. The organic layer was removed water with $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure to give yellow solid (12.43 g, 95%).

^{1}H NMR (CD_3OD, 400Hz) δ: 2.60 (3H, s), 5.80 (2H, s).

1-(2-Hydroxy-4,6-dimethoxy-phenyl)ethanone (3)

K_2CO_3 (20.43 g, 147.8 mmol) was added to a solution of compound **2** (12.43 g, 73.92 mmol) in acetone (200 mL). Dimethyl sulfate (14.0 mL, 20.43 mmol) was added in the reaction vessel with separating three times for 1h and the mixture was stirred at 50 ℃ for 6h. The resulting mixture was added water (300 mL) and extracted with EtOAc (300 mL). The organic layer was dried with $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure to give a white solid (11.11 g, 77%).

^{1}H NMR ($CDCl_3$, 400Hz) δ: 2.60 (3H, s), 3.81 (3H, s), 3.84 (3H, s), 5.91 (1H, d, *J*=2.3Hz), 6.05 (1H, d, *J*= 2.3Hz).

3-(3,4-Dimethoxy-phenyl)-1-(2-hydroxy-4,6-dimethoxy-phenyl)propenone (4)

Compound **3** (11.11 g, 56.65 mmol) and veratraldehyde (11.29 g, 67.95 mmol) were added to a solution of potassium hydroxide (44.48 g, 798.8 mmol) in methanol (250 mL) and stirred at 80 ℃ for 24h. The mixture was acidified until pH 7 using conc. HCl. The solid of mixture was filtered to give a yellow solid (9.60 g, 49%).

^{1}H NMR ($CDCl_3$, 400Hz) δ: 3.83-3.93 (12H, m), 5.96 (1H, d, *J*=2.4Hz), 6.11 (1H, d, *J*=2.4Hz), 6.90 (1H, d, *J*=8.3Hz), 7.22 (1H, dd, *J*=8.3, 2.4Hz), 7.81(2H, m).

2-(3′,4′-Dimethoxyphenyl)-5,7-dimethoxychromen-4-one (5)

Compound **4** (9.60 g, 27.87 mmol) was added to a solution of iodine (0.71 g, 2.79 mmol) in DMSO (150 mL) and stirred at 100 ℃ for 12h. The reaction mixture was quenched with 0.5% $NaHSO_3$ solution (300 mL) and then the solid was filtered to give yellow solid (9.20 g, 96%).

^{1}H NMR ($CDCl_3$, 400Hz) δ: 3.91 (3H, s), 3.95 (3H, s), 3.96 (3H, s), 3.97 (3H, s), 6.38 (1H, d, *J*=2.3Hz), 6.56 (1H, d, *J*=2.3Hz), 6.61 (1H, s), 6.97 (1H, d, *J*=8.6Hz), 7.32 (1H, d, *J*=2.1Hz), 7.52 (1H, dd, *J*=8.6, 2.1Hz).

2-(3,4-Dihydroxy-phenyl)-5,7-dihydroxy-chromen-4-one (6)

Pyridine HCl (15.0 g, 130 mmol) was added to a mixture of Compound **5** (9.20 mL, 26.87 mmol) and water (0.5 mL) and then stirred at 160 ℃ for 14h. The reaction mixture was added water (400 mL) and then cooled to room temperature and stirred for 1h. The solid of reaction mixture was filtered and washed with EtOAc to give a yellow solid (7.24 g, 95%).

^{1}H NMR (DMSO-d_6, 400Hz) δ: 6.15 (1H, d, *J*=2.1Hz), 6.41 (1H, d, *J*=2.1Hz), 6.64 (1H, s) 6.86 (1H, d, *J*=8.3Hz), 7.40 (2H, dd, *J*=8.3, 2.2Hz), 12.94 (1H, s).

2-(2,2-Diphenyl-benzo[1,3]dioxol-5-yl)-5,7-dihydroxy-chromen-4-one (7)

To a stirring mixture of compound **6** (600 mg, 2.10 mmol) in diphenyl ether (20 mL) was added dichlorodiphenylmethane (0.60 mL, 3.14 mmol) and then stirred at 180 ℃ for 30 min. The mixture was cooled to room temperature. Petroleum ether (200 mL) was added to the mixture and the solid formed was filtered. The filtrate was concentrated, and the crude solid was purified by column chromatography using CH_2Cl_2 as eluent to yield a yellow solid (798 mg, 85%).

^{1}H NMR ($CDCl_3$, 400Hz) δ: 6.27 (1H, d, *J*=1.6Hz), 6.41 (1H, d, *J*=1.7Hz), 6.58 (1H, s), 6.99 (1H, d, *J* =8.2Hz), 7.38-7.58 (13H, m), 12.81 (1H, s).

2-(2,2-Diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-7-methoxy-chromen-4-one (8)

Iodomethane (95.1 mg, 0.67 mmol) and K_2CO_3 (92.6 mg, 0.67 mmol) were added to a solution of compound **7** (300 mg, 0.67 mmol) in DMF (5 mL) and stirred at room temperature for 12h. The resulting mixture was added water (30 mL) and extracted with EtOAc (50 mL). The organic layer was washed with brine (30 mL), dried with $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure. The crude material was separated by column chromatography using CH_2Cl_2 as eluent to yield a yellow solid (250 mg, 80%).

^{1}H NMR ($CDCl_3$, 400Hz) δ: 3.86 (3H, s), 6.35 (1H, d, *J*=2.2Hz), 6.45 (1H, d, *J*=2.2Hz), 6.51 (1H, s), 6.99 (1H, d, *J*=8.2Hz), 7.38-7.59 (12H, m), 12.75 (1H, s).

2-(2,2-Diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-7-ethoxychromen-4-one (9)

Compound **9** was synthesized in the same method as compound **8** using iodoethane.

^{1}H NMR ($CDCl_3$, 400Hz) δ: 1.46 (3H, t), 4.12 (2H, q), 6.34 (1H, d, *J*=2.2Hz), 6.43 (1H, d, *J*=2.2Hz), 6.51 (1H, s), 6.99 (1H, d, *J*=8.3Hz), 7.38-7.59 (12H, m), 12.73 (1H, s).

2-(3,4-Dihydroxy-phenyl)-5-hydroxy-7-methoxy-chromen-4-one (10)

A solution of compound **8** (250 mg, 0.54 mmol) in a mixture of AcOH/H_2O (80/20, 60 mL) was refluxed at

120 ℃ for 12h under stirring. The resulting mixture was treated with water (100 mL) and extracted with EtOAc (100 mL). The organic layer was washed $NaHCO_3$ solution (100 mL), dried with $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure. The crude mixture was solidified using CH_2Cl_2 to give a pale yellow solid (120 mg, 74%).

^{1}H NMR (DMSO-d_6, 400Hz) δ: 3.82 (3H, s), 6.28 (1H, d, *J*=2.2Hz), 6.57 (1H, s), 6.63 (1H, d, *J*=8.4Hz), 6.69 (1H, d, *J*=2.2Hz), 7.29 (1H, d, *J*=2.3Hz), 7.35 (1H, dd, *J*=8.4, 2.3Hz).

2-(3,4-Dihydroxy-phenyl)-5-hydroxy-7-ethoxy-chromen-4-one (11)

Compound **11** was synthesized using the same experimental method as Compound **10** using Compound **9**.

^{1}H NMR (DMSO-d_6, 400Hz) δ: 1.33 (3H, t), 4.13 (2H, m), 6.25 (1H, d, *J*=1.8Hz), 6.54 (1H, s), 6.60 (1H, d, *J*=8.5Hz), 6.67 (1H, d, *J*=2.1Hz), 7.27 (1H, s), 7.34 (1H, dd, *J*=8.5, 2.1Hz).

Acetic acid 2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-4-oxo-4H-chromen-7-yl ester (12)

Acetic anhydride (22.5 mg, 0.22 mmol) was added to a solution of Compound **7** (100 mg, 0.22 mmol) in pyridine (10 mL) and stirred at 70 ℃ for 3h. The mixture was acidified to pH 2 with conc. HCl. The resulting mixture was added water (40 mL) and extracted with EtOAc (40 mL). The organic layer was dried with $MgSO_4$ and filtered. The filtrate was concentrated and the crude mixture was separated by column chromatography with CH_2Cl_2/EtOAc (40/1) as eluent to give a yellow solid **12** (71.2 mg, 66%).

^{1}H NMR ($CDCl_3$, 400Hz) δ: 2.33 (3H, s), 6.54 (1H, d, *J*=2.2Hz), 6.58 (1H, s), 6.80 (1H, d, *J*=2.0Hz), 7.00 (1H, d, *J*=8.3Hz), 7.37-7.41 (7H, m), 7.48 (1H, dd), 7.56-7.58 (4H, m), 12.77 (1H, s).

Propionic acid 2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-4-oxo-4H-chromen-7-yl ester (13)

Compound **13** was synthesized in the same manner as Compound **12** using propionic anhydride.

^{1}H NMR ($CDCl_3$, 400Hz) δ: 1.29 (3H, t), 2.64 (2H, m), 6.54 (1H, d, *J*=2.0Hz), 6.58 (1H, s), 6.80 (1H, d, *J*=2.0Hz), 7.00 (1H, d, *J*=9.1Hz), 7.37-7.41 (7H, m), 7.47 (1H, dd, *J*=9.1. 2.0Hz), 7.55-7.58 (4H, m), 12.76 (1H, s).

Butyric acid 2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-4-oxo-4H-chromen-7-yl ester (14)

Compound **14** was synthesized in the same manner as Compound **12** using butyric anhydride.

^{1}H NMR ($CDCl_3$, 400Hz) δ: 1.07 (3H, t), 1.80 (2H, m), 2.58 (2H, m), 6.53 (1H, d, *J*=2.0Hz), 6.58 (1H, s), 6.79 (1H, d, *J*=1.9Hz), 7.00 (1H, d, *J*=8.3Hz), 7.37-7.42 (7H, m), 7.47 (1H, dd), 7.55-7.58 (4H, m), 12.76 (1H, s).

Octanoic acid 2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-4-oxo-4H-chromen-7-yl ester (15)

Compound **15** was synthesized in the same manner as Compound **12** using octanoic anhydride.

^{1}H NMR ($CDCl_3$, 400Hz) δ: 0.91 (3H, m), 1.33 (8H, m), 1.75 (2H, m), 2.59 (2H, t), 6.53 (1H, d, *J*=2.0Hz), 6.58 (1H, s), 6.79 (1H, d, *J*=2.0Hz), 7.00 (1H, d, *J*=8.3Hz), 7.38-7.41 (7H, m), 7.48 (1H, dd, *J*=8.3. 2.0Hz), 7.56-7.58 (4H, m), 12.76 (1H, s).

Acetic acid 2-(3,4-dihydroxy-phenyl)-5-hydroxy-4-oxo-4H-chromen-7-yl ester (16)

To a solution of compound **12** (170 mg, 0.35 mmol) in EtOH/THF (1/1, 20 mL) was added 10% Pd/C (20 mg) at room temperature and then stirred under hydrogen gas for 2 days. The resulting mixture was filtered through celite pad and the filtrate was concentrated under reduced pressure. The crude mixture was separated by column chromatography using CH_2Cl_2/MeOH (10/1) as eluent to give a yellow solid (84.9 mg, 74%).

^{1}H NMR (DMSO-d_6, 400Hz) δ: 2.28 (3H, s), 6.60 (1H, d, *J*=2.0Hz), 6.60 (1H, s), 6.87 (1H, d, *J*=8.4Hz), 6.99 (1H, d, *J*=2.0Hz), 7.44 (2H, m), 9.42 (1H, s), 10.01 (1H, s), 12.99 (1H, s).

Propionic acid 2-(3,4-dihydroxy-phenyl)-3,5-dihydroxy-4-oxo-4H-chromen-7-yl ester (17)

Compound **17** was synthesized using the same method as Compound **16** using Compound **13**.

^{1}H NMR (DMSO-d_6, 400Hz) δ: 0.96 (3H, t), 1.67 (2H, m), 2.58 (2H, m), 6.59 (1H, d, *J*=2.0Hz), 6.81 (1H, s), 6.88 (1H, d, *J*=8.4Hz), 6.99 (1H, d, *J*=2.0Hz), 7.41 (1H, d, *J*=2.3Hz), 7.46 (1H, m), 13.00 (1H, s).

Butyric acid 2-(3,4-dihydroxy-phenyl)-3,5-dihydroxy-4-oxo-4H-chromen-7-yl ester (18)

Compound **18** was synthesized using the same method as Compound **16** using Compound **14**.

^{1}H NMR (DMSO-d_6, 400Hz) δ: 0.96 (3H, m), 1.67 (2H, m), 2.58 (2H, m), 6.59 (1H, d, *J*=2.0Hz), 6.81 (1H, s), 6.88 (1H, d, *J*=8.4Hz), 6.99 (1H, d, *J*=2.0Hz), 7.41 (1H, d, *J*=2.3Hz), 7.46 (1H, m), 13.00 (1H, s).

Octanoic acid 2-(3,4-dihydroxy-phenyl)-3,5-dihydroxy-4-oxo-4H-chromen-7-yl ester (19)

Compound **19** was synthesized using the same method as Compound **16** using Compound **15**.

^{1}H NMR (DMSO-d_6, 400Hz) δ: 0.85 (3H, m), 1.24 (8H, s), 1.62 (2H, m), 2.58 (2H, m), 6.57 (1H, s), 6.87 (2H, t), 6.98 (1H, s), 7.44 (2H, m), 9.39 (1H, s), 10.03 (1H, s), 12.99 (1H, s).

2-(2,2-Diphenylbenzo[d][1,3]dioxol-5-yl)-3,5,7-trihydroxyl-4H-chromen-4-one (20)

To a stirring mixture of quercetin (1.0 g, 3.48 mmol) in

diphenyl ether (30 mL) was added dichlorodiphenylmethane (1.38 mL, 6.96 mmol). The reaction mixture was stirred at 180 ℃ for 30 min. and then cooled to room temperature. Petroleum ether (200 mL) was added to the mixture and the solid formed was filtered. The filtrate was concentrated, and the crude solid was purified by column chromatography using hexane/ EtOAc (4/1) as eluent to yield a yellow solid (0.91 g, 86%).

^{1}H NMR (DMSO-d_6, 400MHz) δ: 6.19 (1H, d, *J*=2.0Hz), 6.47 (1H, d, *J*=2.0Hz), 7.23 (1H, d, *J*=8.3Hz), 7.47 (10H, m), 7.57 (1H, d, *J*=1.7Hz), 7.58 (1H, d, *J*=2.0Hz), 9.68 (1H, s), 10.85 (1H, s), 12.38 (1H, s).

3-(Benzyloxy)-2-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)-5-hydroxy-4H-chromen-4-one (21)

Benzylbromide (0.89 mL, 7.52 mmol) and K_2CO_3 (1.30 g, 9.4 mmol) were added to a solution of compound **20** (3.51 g, 7.52 mmol) in DMF (30 mL) and stirred at room temperature for 12h. The reaction mixture was added water (200 mL) and extracted with EtOAc (300 mL). The organic layer was washed with brine (200 mL), dried with $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and the residue was separated by column chromatography using CH_2Cl_2/EtOAc (20/1) as eluent (2.47 g, 59%).

^{1}H NMR ($CDCl_3$, 400MHz) δ: 5.02 (2H, s), 6.28 (1H, s), 6.36 (1H, s), 6.92 (1H, d, *J*=8.3Hz), 7.25 (5H, m), 7.40-7.60 (12H, m), 12.75 (1H, s).

3-Benzyloxy-2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-7-methoxy-chromen-4-one (22)

Iodomethane (1.40 g, 9.88 mmol) and K_2CO_3 (1.24 g, 8.98 mmol) were added to a solution of compound **21** (5.00 g, 8.98 mmol) in DMF (30 mL) and stirred at room temperature for 12h. The reaction mixture was treated with water (200 mL) and extracted with EtOAc (300 mL). The organic layer was washed with brine (200 mL), dried with $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and the residue was separated by column chromatography using M.C/EtOAc (20:1) as eluent (4.41 g, 86%).

^{1}H NMR ($CDCl_3$, 400MHz) δ: 3.86 (3H, s), 5.03 (2H, s), 6.36 (1H, d, *J*=2.1Hz), 6.41 (1H, d, *J*=2.0Hz), 6.92 (1H, d. *J*=8.3Hz), 7.28 (4H, m), 7.40-7.44 (7H, m), 7.51 (1H, d, *J*=1.6Hz), 7.56-7.61 (5H, m,), 12.69 (1H, s).

3-Benzyloxy-2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-7-ethoxy-chromen-4-one (23)

Compound **23** was synthesized in the same method as Compound **22** using iodoethane.

^{1}H NMR ($CDCl_3$, 400MHz) δ: 1.45 (3H, t, *J*=6.8Hz), 4.10 (2H, q, *J*=6.8Hz), 5.02 (2H, s), 6.38 (2H, m), 6.91 (1H, d, *J*=8.3Hz), 7.11-7.18 (3H, m), 7.27-7.60 (14H , m), 12.66 (1H, s).

3-Benzyloxy-2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-7-propoxy-chromen-4-one (24)

Compound **24** was synthesized in the same method as Compound **22** using iodopropane.

^{1}H NMR ($CDCl_3$, 400MHz) δ: 1.05 (3H, t, *J*=6.7Hz), 1.85 (2H, m), 3.98 (2H, m), 5.03 (2H, s), 6.34 (1H, d, *J*=2.0Hz), 6.39 (1H, d, *J*=2.1Hz), 6.91 (1H, d, *J*=8.4Hz), 7.11-7.18 (3H, m), 7.27-7.60 (14H, m), 12.65 (1H, s).

3-Benzyloxy-7-butoxy-2-(2,2-diphenyl-benzo[1,3] dioxol-5-yl)-5-hydroxy-chromen-4-one (25)

Compound **25** was synthesized in the same method as Compound **22** using iodobutane.

^{1}H NMR ($CDCl_3$, 400MHz) δ: 0.99 (3H, m), 1.51 (2H, m), 1.81 (2H, m), 4.02 (2H, m), 5.02 (2H, s), 6.34 (1H, d, *J*=2.1Hz), 6.39 (1H, d, *J*=2.1Hz), 6.91 (1H, d, *J*=8.3Hz), 7.12-7.18 (3H, m), 7.39-7.60 (14H, m), 12.66 (1H, s).

3-Benzyloxy-2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-7-octyloxy-chromen-4-one (26)

Compound **26** was synthesized in the same method as Compound **22** using iodooctane.

^{1}H NMR ($CDCl_3$, 400MHz) δ: 0.89 (3H, m), 1.33 (8H, m), 1.45 (2H, m), 1.82 (2H, m), 4.01 (2H, m), 5.02 (2H, s), 6.34 (1H, d, *J*=2.0Hz), 6.38 (1H, d, *J*=2.0Hz), 6.91 (1H, d, *J*=8.3Hz), 7.11-7.18 (3H, m), 7.39-7.60 (14H, m), 12.64 (1H, s).

2-(3,4-Dihydroxy-phenyl)-3,5-dihydroxy-7-methoxy-chromen-4-one (27)

A solution of compound **22** (4.41 g, 7.73 mmol) in a mixture of AcOH/H_2O (80/20, 100 mL) was refluxed at 140 ℃ for 6h. The reaction mixture was cooled to room temperature and water (200 mL) was added, extracted with EtOAc (200 mL). The organic layer was washed with $NaHCO_3$ solution (100 mL), dried with $MgSO_4$, and then filtered. The filtrate was concentrated under reduced pressure.

The residue was treated with EtOH/THF (1:1, 30 mL), and then with 10% Pd/C (30 mg) at room temperature. Then the reaction mixture was stirred under hydrogen gas for 20h. The mixture was filtered through celite pad and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography using CH_2Cl_2/MeOH (4:1) as eluent to give a yellow solid (191 mg, 76%).

^{1}H NMR (DMSO-d_6, 400MHz) δ: 3.87 (3H, s), 6.36 (1H, d, *J*=2.4Hz), 6.70 (1H, s), 6.91 (1H, d, *J*=8.4Hz), 7.59 (1H, d, *J*=8.8Hz), 7.73 (1H, s).

2-(3,4-Dihydroxy-phenyl)-3,5-dihydroxy-7-ethoxy-chromen-4-one (28)

Compound **28** was synthesized in the same method as in

Compound **27** using Compound **23**.

^{1}H NMR (DMSO-d_6, 400MHz) δ: 1.33 (3H, t, *J*=6.8Hz), 4.13 (2H, q, *J*=6.8Hz), 6.28 (1H, d, *J*=2.1Hz), 6.65 (1H, d, *J*=2.0Hz), 6.85 (1H, d, *J*=8.5Hz), 7.55 (1H, m), 7.69 (1H, d, J=2.1Hz).

2-(3,4-Dihydroxy-phenyl)-3,5-dihydroxy-7-propoxy-chromen-4-one (29)

Compound **29** was synthesized in the same method as in Compound **27** using Compound **24**.

^{1}H NMR (DMSO-d_6, 400MHz) δ: 0.96 (3H, t, *J*=6.7Hz), 1.74 (2H, m), 4.03 (2H, t, *J*=6.7Hz), 6.29 (1H, d, *J*=2.1Hz), 6.66 (1H, d, *J*=2.1Hz), 6.85 (1H, d, *J*=8.5Hz), 7.55 (1H, dd, *J*=8.5, 2.1Hz), 7.69 (1H, d, *J*=2.1Hz).

7-Butoxy-2-(3,4-dihydroxy-phenyl)-3,5-dihydroxy-chromen-4-one (30)

Compound **30** was synthesized in the same method as in Compound **27** using Compound **25**.

^{1}H NMR (DMSO-d_6, 400MHz) δ: 0.92 (3H, t, *J*=6.9Hz), 1.41 (2H, m), 1.68 (2H, m), 4.06 (2H, m), 6.29 (1H, d, *J*=2.1Hz), 6.67 (1H, d, *J*=2.2Hz), 6.86 (1H, d, *J*=8.5Hz), 7.54 (1H, m), 7.69 (1H, d, *J*=2.2Hz), 12.44 (1H, s).

2-(3,4-Dihydroxy-phenyl)-3,5-dihydroxy-7-octyloxy-chromen-4-one (31)

Compound **31** was synthesized in the same method as in Compound **27** using Compound **26**.

^{1}H NMR (DMSO-d_6, 400MHz) δ: 0.82 (3H, t, *J*=7.0Hz), 1.35 (12H, m), 1.71 (2H, m), 4.05 (2H, t, *J*=6.8Hz), 6.28 (1H, d, *J*=2.0Hz), 6.66 (1H, d, *J*=2.0Hz), 6.85 (1H, d, *J*=8.5Hz), 7.54 (1H, m), 7.69 (1H, d, *J*=2.0Hz), 12.45 (1H, s).

Acetic acid 3-benzyloxy-2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-4-oxo-4H-chromen-7-yl ester (32)

Acetic anhydride (0.12 mL, 1.26 mmol) was added to the solution of Compound **21** (700 mg, 1.26 mmol) in pyridine (10 mL) and stirred at 70 ℃ for 3h. The reaction mixture was acidified with 1N HCl (40 mL) and then extracted with EtOAc (100 mL), dried with $MgSO_4$, and filtered. The filtrate was concentrated under reduced pressure and the residue was separated by column chromatography using CH_2Cl_2/EtOAc (60/1) as eluent to give a yellow solid (578 mg, 76%).

^{1}H NMR ($CDCl_3$, 400MHz) δ: 2.32 (3H, s), 5.03 (2H, s), 6.53 (1H, d, *J*=2.0Hz), 6.74 (1H, d, *J*=2.0Hz), 6.91 (1H, d, *J*=8.4Hz), 7.14-7.60 (17H, m), 12.71 (1H, s).

Propionic acid 3-benzyloxy-2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-5-hydroxy-4-oxo-4H-chromen-7-yl ester (33)

Compound **33** was synthesized in the same method as Compound **32** using propionic anhydride.

^{1}H NMR ($CDCl_3$, 400MHz) δ: 1.29 (3H, t), 2.64 (2H, q), 5.04 (2H, s), 6.54 (1H, d, *J*=2.0Hz), 6.76 (1H, d, *J*=2.0Hz), 6.93 (1H, d, *J*=8.3Hz), 7.12-7.25 (3H, m), 7.40-7.61 (14H, m), 12.71 (1H, s).

Butyric acid 3-benzyloxy-2-(2,2-diphenyl-benzo[1,3]diox-ol-5-yl)-5-hydroxy-4-oxo-4H-chromen-7-yl ester (34)

Compound **34** was synthesized in the same method as Compound **32** using butyric anhydride.

^{1}H NMR ($CDCl_3$, 400MHz) δ: 1.06 (3H, t, *J*=7.0Hz), 1.81 (2H, m), 2.58 (2H, t, *J*=6.8Hz), 5.04 (2H, s), 6.54 (1H, d, *J*=1.9Hz), 6.74 (1H, d, *J*=1.9Hz), 6.92 (1H, d, *J*=8.3Hz), 7.11-7.20 (4H, m), 7.29-7.60 (13H, m), 12.70 (1H, s).

Octanoic acid 3-benzyloxy-2-(2,2-diphenyl-benzo[1,3]di-oxol-5-yl)-5-hydroxy-4-oxo-4H-chromen-7-yl ester (35)

Compound **35** was synthesized in the same method as Compound **32** using octanoic anhydride.

^{1}H NMR ($CDCl_3$, 400MHz) δ: 1.06 (3H, t, *J*=7.1Hz), 1.81 (2H, m), 2.58 (2H, t, *J*=6.9Hz), 5.04 (2H, s), 6.54 (1H, d, *J*=1.9Hz), 6.74 (1H, d, *J*=1.9Hz), 6.92 (1H, d, *J*=8.3Hz), 7.11-7.20 (4H, m), 7.29-7.60 (13H, m), 12.70(1H, s).

Acetic acid 2-(3,4-dihydroxy-phenyl)-3,5-dihydroxy-4-oxo-4H-chromen-7-yl ester (36)

To a solution of compound **32** (570 mg, 0.95 mmol) in EtOH/THF (1/1, 30mL) was added 10% Pd/C (60 mg) at room temperature and then stirred under hydrogen gas for 2 days. The resulting mixture was filtered through celite pad and the filtrate was concentrated under reduced pressure. The residue was separated by column chromatography using CH_2Cl_2/MeOH (40/1) as eluent to give a yellow solid (144 mg, 44%).

^{1}H NMR (DMSO-d_6, 400MHz) δ: 2.67 (3H, s), 6.57 (1H, d, *J*=2.00Hz), 6.87 (1H, d, *J*=8.5Hz), 6.97 (1H, d, *J*=2.0Hz), 7.56 (1H, dd, *J*=8.5, 2.2Hz), 7.70 (1H, d, *J*=2.2Hz), 12.55 (1H, s).

Propionic acid 2-(3,4-dihydroxy-phenyl)-3,5-dihydroxy-4-oxo-4H-chromen-7-yl ester (37)

Compound **37** was synthesized in the same method as in compound **36** using compound **33**.

^{1}H NMR (DMSO-d_6, 400MHz) δ: 1.12 (3H, t, *J*=7.0Hz), 2.63 (2H, q, *J*=7.0Hz), 6.57 (1H, d, *J*=1.9Hz), 6.86 (1H, d, *J*=8.3Hz), 6.97 (1H, d, *J*=2.0Hz), 7.56 (1H, d, *J*=2.1Hz), 7.70 (1H, d, *J*=2.2Hz), 12.55 (1H, s).

Butyric acid 2-(3,4-dihydroxy-phenyl)-3,5-dihydroxy-4-oxo-4H-chromen-7-yl ester (38)

Compound **38** was synthesized in the same method as in compound **36** using compound **34**.

^{1}H NMR (DMSO-d_6, 400MHz) δ: 0.96 (3H, t, *J*=6.8Hz), 1.67 (2H, m), 2.58 (2H, m), 6.56 (1H, d, *J*=1.9Hz), 6.87 (1H, d, *J*=8.5Hz), 6.97 (1H, d, *J*=2.0Hz), 7.57 (1H, m), 7.71 (1H, d, *J*=2.1Hz), 9.30 (1H, s), 9.66 (2H, s), 12.55 (1H, s).

Octanoic acid 2-(3,4-dihydroxy-phenyl)-3,5-dihydroxy-4-oxo-4H-chromen-7-yl ester (39)

Compound **39** was synthesized in the same method as in compound **36** using compound **35**.

^{1}H NMR (DMSO-d_6, 400MHz) δ: 0.85 (3H, t, *J*=7.1Hz), 1.30 (10H, m), 1.60 (2H, m), 2.57 (2H, t, *J*=6.9Hz), 6.55 (1H, d, *J*=2.0Hz), 6.87 (1H, d, *J*=8.5Hz), 6.97 (1H, d, *J*=2.0Hz), 7.57 (1H, m), 7.71 (1H, d, *J*=2.2Hz), 9.30 (1H, s), 9.66 (2H, s), 12.55 (1H, s).

B16 Melanoma Cell Assay

Measurement of cell cytotoxicity was performed, according to a cytotox 96 non-radioactive cytotoxicity assay in PROMEGA, B16-F0 cell seeded in 96-plate well at 1.0 × 10^4 cells per well. After 24h, test compounds and vehicle control are added to the appropriate wells so that the final volume in each well is 100 μl. Absorbance was measured at 490 nm by using a microplate reader. Each experiment was repeated three times. Cell cytotoxicity was expressed as a percentage of the proliferation measured in control cells treated with vehicle without the sample materials.

When B16-F0 cells were treated with luteolin and its derivatives, luteolin exhibited the highest toxicity. The alkyl-substituted derivatives of luteolin were found to have lower toxicity than the acyl-substituted derivatives, with compound **10** showing the lowest toxicity (*Fig.* 4).

Quercetin and all its alkyl- and acyl-substituted derivatives have been shown to have similar degrees of toxicity. Acyl-substituted derivatives of quercetin were found to be more toxic than alkyl-substituted derivatives (*Fig.* 5).

Quercetin and its gallic acid conjugates were found to have similar toxicity, with 3′-gallic acid conjugate showing the lowest toxicity (*Fig.* 6).

Measurement of cell proliferation was performed, according to a previously described method,[18] B16-F0 cell seeded in 96-plate well at 1.0 × 10^4 cells per well. After 24h, test compounds and vehicle control are added to the appropriate wells so that the final volume in each well is 100 μl. Absorbance was measured at 490 nm by using a microplate reader. Each experiment was repeated three times. Cell proliferation was expressed as a percentage of the proliferation measured in control cells treated with vehicle without the sample materials.

Cell proliferation was inhibited by luteolin and its alkyl- and acyl-substituted derivatives. Acyl-substituted luteolin derivatives were found to have greater inhibitory effects on cell proliferation than the alkyl-substituted derivatives (*Fig.* 7).

Quercetin and its alkyl- and acyl-substituted derivatives inhibited cell proliferation. The alkyl-substituted derivatives of quercetin had a greater inhibitory effect than the acyl-substituted derivatives, with compound **29** showing the greatest inhibitory effect on cell proliferation (*Fig.* 8).

Quercetin and all its mono-gallic acid conjugates showed

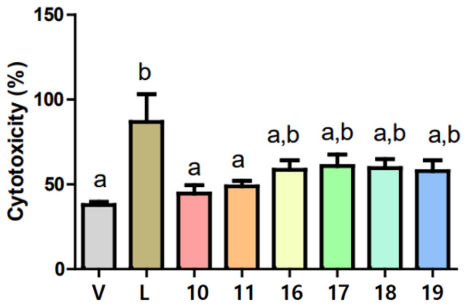


***Figure* 4.** Cytotoxicity of luteolin and its alkyl (**10**, **11**) and acyl (**16-19**) derivatives in B16 melanoma cells (V: vehicle, L: luteolin). The data is reported as the mean±SEM (n=4). Different letters above bars indicate a significant difference by one-way ANOVA followed by Tukey's test (P<0.05).

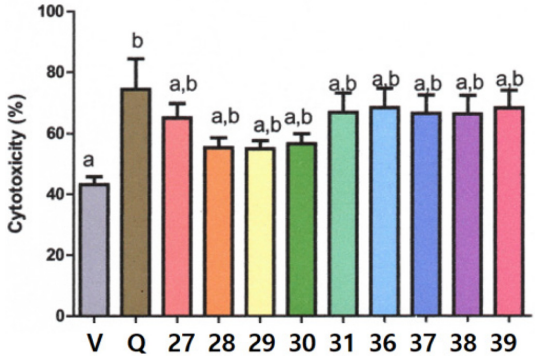


***Figure* 5.** Cytotoxicity of quercetin and its alkyl (**27-31**) and acyl (**36-39**) derivatives in B16 melanoma cells (V: vehicle, Q: quercetin). The data is reported as the mean±SEM (n=4). Different letters above bars indicate a significant difference by one-way ANOVA followed by Tukey's test (P<0.05).

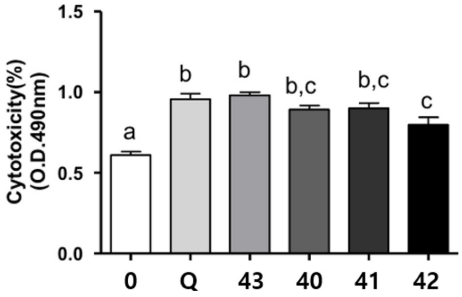


***Figure* 6.** Cytotoxicity of quercetin and its gallic acid conjugates in B16 melanoma cells (0: vehicle, Q: quercetin). The data is reported as the mean±SEM (n=4). Different letters above bars indicate a significant difference by one-way ANOVA followed by Tukey's test (P<0.05).

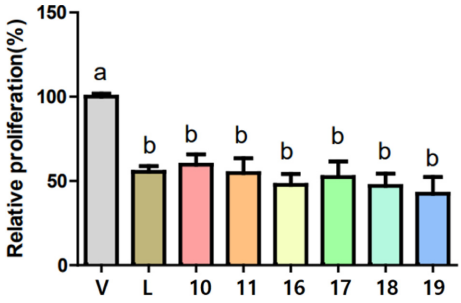


***Figure* 7.** Proliferation of luteolin and its alkyl (**10**, **11**) and acyl (**16-19**) derivatives in B16 melanoma cells (V: vehicle, L: luteolin). The data is reported as the mean±SEM (n=4). Different letters above bars indicate a significant difference by one-way ANOVA followed by Tukey's test (P<0.05).

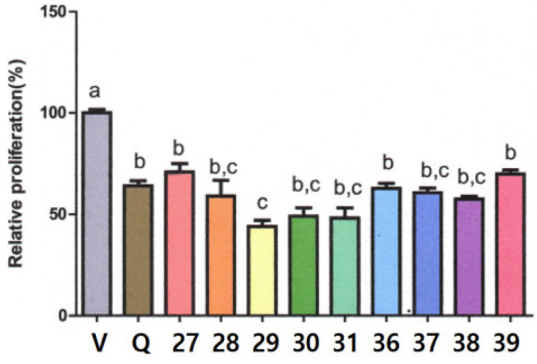


***Figure* 8.** Proliferation of quercetin and its alkyl (**27-31**) and acyl (**36-39**) derivatives in B16 melanoma cells (V: vehicle, Q: quercetin). The data is reported as the mean±SEM (n=4). Different letters above bars indicate a significant difference by one-way ANOVA followed by Tukey's test (P<0.05).

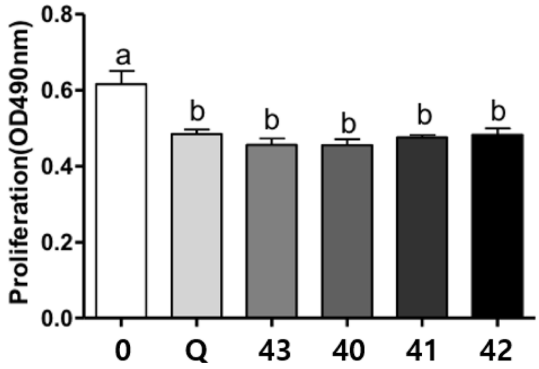


***Figure* 9.** Proliferation of quercetin and its gallic acid conjugates in B16 melanoma cells (0: vehicle, Q: quercetin). The data is reported as the mean±SEM (n=4). Different letters above bars indicate a significant difference by one-way ANOVA followed by Tukey's test (P<0.05).

similar inhibitory effects on cell proliferation without significant differences (*Fig.* 9).

RESULTS AND DISCUSSION

In this study, two alkylated and five acylated derivatives of the 7-hydroxyl group were synthesized from luteolin along with five alkylated and four acylated derivatives of the 7-hydroxyl group of quercetin. The process of selectively acylating the 7-hydroxyl groups of quercetin and luteolin was presented for the first time.

Acylated derivatives of the 7-hydroxyl group of luteolin showed relatively low cytotoxicity in B16 melanoma cells compared with luteolin. Thus, the 7-hydroxyl group of luteolin appeared to play an important role in inducing cytotoxicity. In addition, the inhibitory effect on cell proliferation was excellent for the acylated derivatives of the 7-hydroxyl group of luteolin, and it was observed that the effect increased as the carbon number of the acyl-group increased.

The alkylated derivatives of quercetin showed relatively low cytotoxicity and high cell proliferation inhibitory effects compared with the acylated derivatives. Compound **29** showed the lowest cytotoxicity and the highest cytostatic effect.

The alkylated derivatives of luteolin and quercetin showed lower cytotoxicity than the acylated derivatives. Regarding the inhibitory effect on cell proliferation, luteolin-acylated and quercetin-alkylated derivatives presented better effects.

All quercetin and the four quercetin gallic acid coupling compounds had similar toxicity and proliferation inhibitory effects on B16 melanoma cells.

In addition, a difference in the cytotoxicity and proliferation inhibition at each substituted hydroxyl position was observed, and the gallic acid coupling compound substituted with the 3′-hydroxyl group had the highest effect of inhibiting the cytotoxicity of melanoma cells.

CONCLUSION

This study focused on the synthesis of selectively alkylated and acylated derivatives of the 7-hydroxyl groups of luteolin and quercetin.

Four 7-hydroxyl acylated and two alkylated derivatives of luteolin were synthesized, along with four acylated and five alkylated derivatives of quercetin.

In addition, the cytotoxicity and cell proliferation of quercetin and luteolin derivatives, and quercetin gallic acid-coupled compounds were studied in B16 melanoma cells.

All the flavonoid compounds used in this study were

toxic to B16 melanoma cells and inhibited cell proliferation. Based on the results of cytotoxicity and inhibition of cell proliferation in B16 melanoma cells, the derivatives of luteolin and quercetin have great potential as therapeutic agents for the skin cancer-melanoma type.

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