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Transglycosylation and biochemical characterization of epigallocatechin gallate glucosides using dextransucrase

Dextransucrase를 이용한 EGCG (Epigallocatechin gallate glucosides) 배당체 생성 및 생화학적 특성 연구

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Abstract

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Green tea (*Camellia sinensis*) has attracted significant attention, both in the scientific and in consumer communities, because of its health benefits for a variety of disorders, ranging from cancer to weight loss. Epigallocatechin gallate (EGCG) is the most abundant catechin and the main polyphenolic components of green tea. Although EGCG possess various biological activities but EGCG has limited for application in functional food, cosmetic because of its low solubility. In this study epigallocatechin gallate glucosides (EGCG-Gs) were synthesized by acceptor reaction using dextransucrase from *Leuconostoc mesenteroides* B-1299CB expressed in *E. coli* and analyzed their solubility. The solubility was measured by HPLC using C_{18} column. Novel compounds have shown 42 to 347-folds higher solubility than that of EGCG, especially the solubility of compound 6 (EGCG-5'-O-a-D-glucopyranoside) was 1878.47 mM. With this, glucosylated

EGCG could be utilized highly in various fields. Additionally While EGCG being studied as cosmetic materials for brightening effect, in this study EGCG and its glucosides activated human tyrosinase expressed in *E.coli* and produce melanin unlike mushroom tyrosinase. In biofunctionally, EGCG-Gs have less cell cytotoxicity on epidermal cells than it of EGCG, therefore EGCG-Gs could be treated up to 200 μ M. In addition, EGCG-Gs have higher inhibition ability to produce nitric oxide than EGCG up to 68% in Raw 264.7 cells related with skin aging. Melanin synthesis in B16F10 cells is also inhibited up to 40% (compound 4, EGCG-7,5',4''-O-α-D-glucopyranoside).

Keywords: EGCG, Acceptor reaction, human tyrosinase, melanin, NO assay
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Introduction

(−)−Epigallocatechin gallate (EGCG), a catechin polyphenol compound, represents the main ingredient of green tea extract. It has various functional activities such as anti cancer [1], antioxidant [2], antibacterial effects [3] and strong inhibition ability to mushroom tyrosinase [4]. Furthermore improvement of endothelial function and insulin sensitivity has been studied [5]. To utilize this positive biological characteristic there are various research is underway in cosmetic, pharmaceuticals, and food industries. Although EGCG possess various biological activities but EGCG has limited for application in functional food, cosmetic because of its low solubility, stability, and browning effect [6, 7].

Dextransucrase produced from *Leuconostoc mesenteroides* belongs to the family of transglycosylase that catalyzes the polymerization of dextran from sucrose [8]. Glucosyltransferases (GTFs) are enzymes that synthesize either dextrans or glucans, catalyzing sucrose as a substrate [9]. It is well known that dextransucrases catalyze the transfer of glucosyl units from sucrose to other carbohydrates. This reaction is the so−called acceptor reaction, and the added carbohydrates are called acceptors [10]. GTFs can form diverse glycosidic linkages by differing from one another by one or more glucose residues [11]. Therefore it is possible to utilize dextranansucrases to synthesize new kinds of carbohydrates by the acceptor reaction.

Through the previous study about acceptor reaction of EGCG found that improving of solubility and stability and also manifested a higher degree of browning resistance than was found in EGCG. [12, 13]. Glycosylation is not always functionally positive effect. The EGCG glycosides are more stable than EGCG in aqueous solutions, but exhibited decreasing antioxidant activity in the DPPH radical-scavenging assay [14]. Also, other flavonols, Nugroho, A et al. conducted the flavonol glycosides of quercetin or kempferol were found to be less active than their corresponding aglycones [15].

The human body posses self defense system which maintains homeostasis by distinguishing the invasion of various pathogens and removing it [16]. Immune response is regulated by promoting or suppression. Depending on whether immune system promoted or suppressed there could be inflammation, allergy or autoimmune diseases. [17, 18] When pathogens enter the body, cytokines such as nitric oxide (NO) and tumor necrosis factor-a (TNF-a) are produced and conduct an important role to mechanism of biological defense [19]. However when it is hyper secreted by lipopolysaccharide (LPS) it causes DNA mutation or neural damage by inflammation. LPS is stimulated by UV and synthesis of NO affect to skin aging [20, 21]. Therefore proper production of NO and TNF-a by macrophage carries out key role to regulate the immune system. Various polyphenol compounds including EGCG have high inhibition activity to produce NO [22].

Melanin is an important skin pigment to protect skin damage from UV and various other types of ionizing radiation [23]. Tyrosinase is an enzyme catalyzing the rate-limiting reaction in melanin biosynthesis and the core enzyme in melanogenesis [24]. The enzyme has two different reaction mechanisms. The first one is hydroxylation of monophenol to o-diphenol (monophenolase), and the second is oxidation of o-diphenol to o-quinone (diphenolase). Melanin is produced at the last step by polymerizing [25]. There are various materials of nature studied to inhibit or activate melanogenesis. More research about inhibition of tyrosinase is underway rather than about activation. There are some research that quercetin activates the production of melanin in melanoma cells [26] and various fatty acids also activate tyrosinase [27]. However, much research is under way to inhibit the activity of tyrosinase in most cases.

In this study, we report the synthesis of EGCG glycosides by transglycosylation using dextransuctase. The glycosylated epigallocatechin gallate derivatives were screened for biological activities, including epidermal cell cytotoxicity, anti-inflammatory effect and melanin production in B16F10 melanoma cells. Furthermore human tyrosinase was expressed from *E.coli* and anlyzed melanin synthesis activity of EGCG and EGCG-Gs.

Materials and Methods

1. Dextransucrase activity assay

Enzyme activity was assayed via the incubation of enzyme for different reaction periods (1, 3, 5, 7, 9, 12, 15, 20 min) at 28 ℃ with 100 mM sucrose as a substrate, using 20 mM sodium acetate buffer (pH 5.2). The standard assay mixtures consisted of 200 μl of 200 mM sucrose and 20 μl of an enzyme solution. Each of enzyme reaction samples was spotted on TLC silica gel 60 F_{254} plate (Merck Co.) and then the TLC plate was developed twice in acetonitrile/water (85:15, v/v) solvent system. The plate was dyed by dipping into 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5%(v/v) H_2SO_4 in methanol, followed by 5 min of heating at 121 ℃. After that, each of carbohydrates was visualized. The quantity of fructose released from sucrose was calculated by AlphaEaseFC 4.0 Program (Alpha Inotech, San Leandro, CA, USA), using standard materials. One unit of dextransucrase activity, for the purpose of this study, was defined as the quantity of enzyme required to produce 1 μmol of fructose per min at 28 ℃ and a pH of 5.2, in 20 mM sodium acetate buffer.

2. Glucosylation of EGCG

Glucosylation was conducted in reaction mixture containing

80 mM EGCG, 400mM sucrose, and BF563 dextransucrase (8 U/ml). It was incubated at 28 ◦C for 18 h, after which sucrose had been depleted. the reaction mixture was then kept at 80 ℃ water bath for 8 min to halt the enzyme reaction.

Analysis of acceptor reaction products using thin layer chromatography (TLC)

TLC was conducted at room temperature using silica gel 60 F_{254} TLC plates (Merck Co.). 1 μl of a reaction mixture was spotted onto a lane in a silica gel plate, and the plate was then developed using a solvent mixture of ethyl acetate- acetic acid water (3:1:1, v/v/v). The developed plate was then dried and visualized as previously described.

3. Purification using MPLC

EGCG reaction mixture was filtered (0.45 μm) and purified by MPLC. The column was PuriFlash Column (50 μ C18 STD/450 g). The column was eluted with 100% water and flow rate was 30 ml/min for 90 min to remove saccharides and DMSO, then eluted with 82% water and 18% acetonitrile for 60 min to collect EGCG glycosides. The eluent (EGCG glycosides) was concentrated by rotary evaporator at 45 ℃ and freezing dried.

4. Purification using HPLC

The reaction mixture removed saccharides and DMSO was prepared in water (95%) and acetonitrile (5%) in concentration of 200 mg/ml. Purification of EGCG glucosides were performed

using WATERS 2545 Binary Gradient Module (Pump), 2767 sample manager (Injector), 2998 PDA detector HPLC system equipped with a Sunfire Prep OBD™ C18 (19 mm × 100 mm, 5 ㎛) column and multiple wavelength detector. Mobile phase A was Acetonitrile and mobile phase B was 0.025% HFBA in water. The flow rate was 17 mL/min. The detector was set at 254nm and injection volume was 341 ㎕(Table 1.).

Table 1. Prep condition of HPLC

<table>
<tr><td>☐system</td><td>WATERS 2545 Binary Gradient Module (Pump), 2767 sample manager (Injector), 2998 PDA detector</td></tr>
<tr><td>☐column</td><td>Sunfire Prep OBD™ C18 (19 mm × 100 mm, 5 ㎛)</td></tr>
<tr><td>☐temperature</td><td>Room temperature</td></tr>
<tr><td>☐solvent</td><td>(A) Acetonitirle
(B) 0.025% HFBA</td></tr>
<tr><td>gradient</td><td>
<table>
<tr><th>time (min)</th><th>A(%)</th><th>B(%)</th></tr>
<tr><td>20</td><td>5</td><td>95</td></tr>
<tr><td>20.01</td><td>6</td><td>94</td></tr>
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<tr><td>63.01</td><td>12</td><td>88</td></tr>
<tr><td>70</td><td>12</td><td>88</td></tr>
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</td></tr>
<tr><td>☐flow rate</td><td>1 7 mL/min</td></tr>
<tr><td>☐injection</td><td>341 ㎕</td></tr>
<tr><td>☐absorbance wave</td><td>254 nm</td></tr>
</table>

5. Expression of human tyrosinase in *E.coli*

Human tyrosinase was expressed in *E. coli* as a His-tag fusion protein using the pET-28a expression system based on amino acid sequence of human tyrosinase (GenBank Accession No. AH003020.2). The coding region was cloned into the EcoR1 and NotI restriction sites of the pET-28 a(+)expression vector and thereby fused to the C terminus of the histidine coding region. Overnight cultures of *E. coli* BL21 (DE3), containing the fusion cDNA clone, were grown overnight pET28a-NS2B-NS3pro was transformed into E. coli BL21(DE3) which was then grown in LB supplemented with kanamycin (50 lg ml-1) at 37 ℃. Cells were induced with 0.5 mM IPTG when the OD600 reached 0.5. Induced cellswere kept at 16 ℃ for 12 h at 200 rpm. Cells were collected by centrifugation (80009g for 30 min at 4 ℃), resuspended in 50 mM Tris/HCl (pH 7.0), and lysed via sonication. After centrifugation (12,000xg for 30 min), the cell lysate was loaded onto 8 ml Ni-agarose resin. Proteins were eluted from the column with elution buffer (50 mM Tris/HCl, 30 mM NaCl, 500 mM imidazole, pH 8). Fractions containing pure protein were pooled, concentrated,
and dialyzed against 50 mM Tris/HCl (pH 7). The concentration of protein was determined using the Bio-Rad protein assay. Purified protein was confirmed by 12% SDS-PAGE

6. Human tyrosinase activity

5 μL of human tyrosinase expressed from E.coli was reacted with 3.3 mM L-DOPA and EGCG and EGCG glucosides (250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM) were added as activator. Ptassium phosphate buffer (450 mM, pH 6.8) containing copper sulfate pentahydrate (5 mM) was used to create proper pH and reaction condition. The mixture was reacted for 80 min at 37℃. After then, the absorbance value at 475 nm was read using spectraphotometer.

7. Water Solubility Analysis

EGCG glycosides solubility in water was measured by HPLC. EGCG glycosides were mixed in 1 μL of water in an eppendorf tube until precipitation, at room temperature. Then the mixture was diluted to check the maximum concentration.

High performance liquid chromatography analysis

A Waters Associates liquid chromatography, model WATERS 2545, equipped with a Binary Gradient Module (Pump), sample manager (Injector), 2998 PDA detector.

The column system consisted of a C18 5 μm 4.6x100 mm column packed with SunFire packing. The mobile phase was acetonitrile and water (5:95~8:92,v/v). Operating conditions were flow rate: 1.2 ml/min, absorbance: 258 nm and injection volume was 20 ul. EGCG was prepared as a standard by placing 20 mg of EGCG in a 1 ml of water and diluted to 2 mg/ml.

8. Assay of Cell Viability

Raw 264.7 cells and B16F10 cells were harvest and re-suspended to desired final concentration to seed in100 μL (96 well plate) of growth medium (DMEM). Optimal cell seeding numbers of Raw cell is 2.0 $\times 10^4$ /well (100 μL) and B16F10 cell is 0.5 $\times 10^4$/well (100 μL) respectively. Optimal cell seeding numbers vary widely, though are typically between 5,000 - 40,000 cells per well and must be determined empirically. 100 μL of cell suspension per well was seed. The cells grow overnight in a cell culture incubator (37° C, 5% CO_2). After incubation, samples to be tested for cytotoxicity were treated by concentration with serum-free DMEM. The group untreated sample was considered 100% cell viability relatively as control and then alive cell was determined their cell viability. It was expressed with a percentage after sample treatment. The highest concentration of sample treatment was 800 μM and than diluted by 1/2 to 0.39 μM by pipette (800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.08, 1.54, 0.78, 0.39 μM). After treatment, the plate was incubated at previous condition overnight. Cell state was checked frequently using microscope.
Cell viability was determined using the EZ-Cytox (DoGen Life science, genetic engineering). At the end of the treatment period, 100 μL of water soluble tetrazolium (WST) and DMEM medium (1:9,v/v), was added to each well. The plates were placed at 37° C for 2 h in the dark and the absorbance at 450 nm was read using spectraphotometer microplate reader. Usually, four replicate wells were measured for each group to

be tested. Wells containing medium but no sample treatment served as controls.

9. Raw 264.7 cell NO assay

1)Cell Culture and Treatment

RAW 264.7, a murine monocyte/macrophage cell line, was obtained from the Korean Cell Line Bank. To study the effects of EGCG glucosides on NO production, cells were cultured in 96 well plate with DMEM supplemented with 10% FBS and 1% antibiotics (peniciline) under 5% CO2 at 37° . After incubation for 24 hr, 1 μg/mL of LPS and EGCG glucosides were added except for control wells and incubated for another 24 hr. 100 μM of Indomethacine was used as positive control. The samples were dissolved in distilled water on the day of experiment and diluted with serum-free DMEM into appropriate concentrations.

2)NO inhibition measurement

To quantify the nitric oxide released from Raw cells, 80 μL of media containing nitric oxide was moved to other new 96 well plate. 0.1 μM of Sodium nitric oxide () was diluted to make a calibration curve. Griess reagent (80 μL) was added and the mixture was reacted for 20 min at room temperature until the color changes. The optical density value of the mixture was detected at 540 nm using spectraphotometer microplate reader. Griess reagent: 1. 1% sulfanilamide (w/w) in 5% phosphoric acid (w/w), 2. 0.1% naphthylethylenediamine (w/w). Mix 1 and 2 in equal amount.

10. B16F10 cell melanin synthesis assay

1)Cell Culture and Treatment

B16F10 obtained from the Korean Cell Line Bank is a mouse melanoma cell line that was established from a black-brown malignant melanoma. B16F10 were purchased from Cell Systems. B16F10 cells were cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin. They were grown in a humidified atmosphere with 5% CO2 at 37° C. Cell plating densities were arranged that those cells were in log phase of growth for the duration of incubation with drug. Subcultures of cells were plated at a density of 0.5×10^4 cells/well. Approximately 24 h later, fresh medium containing 500 nM α-MSH and 25 μM of EGCG glycosides were added. Cells were harvested for 3 days after drug addiction. EGCG glycosides were dissolved in distilled water. In some experiments, the transcriptional inhibitor kojic acid (100 μg/ml), and α,β-arbutin (2 mM) were added as standard inhibitor.

2)Melanin assay

Released and cellular melanin content was measured by absorbance measurement. The media containing melanin was transferred to 96 well plate and read at 405 nm. Then cell lysis was carried out using 100 μ L of 1 N sodium hydroxide for 1 h to measure cellular melanin. Cellular melanin was also transferred to 96 well plate and detected at 405 nm.

3)Quantification of cell protein-Lowry assay

Raw 264.7 cells and B16F10 cells protein content was

measured by Lowry assay to calibrate nitric oxide and melanin synthesis inhibition of EGCG glucosides. Cells were washed twice with PBS buffer after removal of existing medium. Cell lysis was conducted using 30 μL of sodium hydroxide (1N) for 1~2 hr in the microplate shaker. 5 μL of sodium hydroxide buffer of the lysed cell was transferred to new 96 well plate. 1 mg/mL of bovine serum albumin (BSA) in distilled water was used as standard protein to calculate the quantity of cell protein. Reagent S and A was mixed together (1:50, v/v) and 25 μL of the mixture was added into well. Reagent B (200 μL) was added at last and wait until color changes for 20 min. (Bio-Rad DC Protein Assay). The absorbance at 750 nm was read using spectraphotometer microplate reader.

Results and Discussions

1. Purification of EGCG and EGCG-Gs

AR2 removed DMSO and saccharides after MPLC purification was separated by HPLC to single compound (Figure 2.). Table shows 5 novel compounds structure and yields of purification (Table 2.).

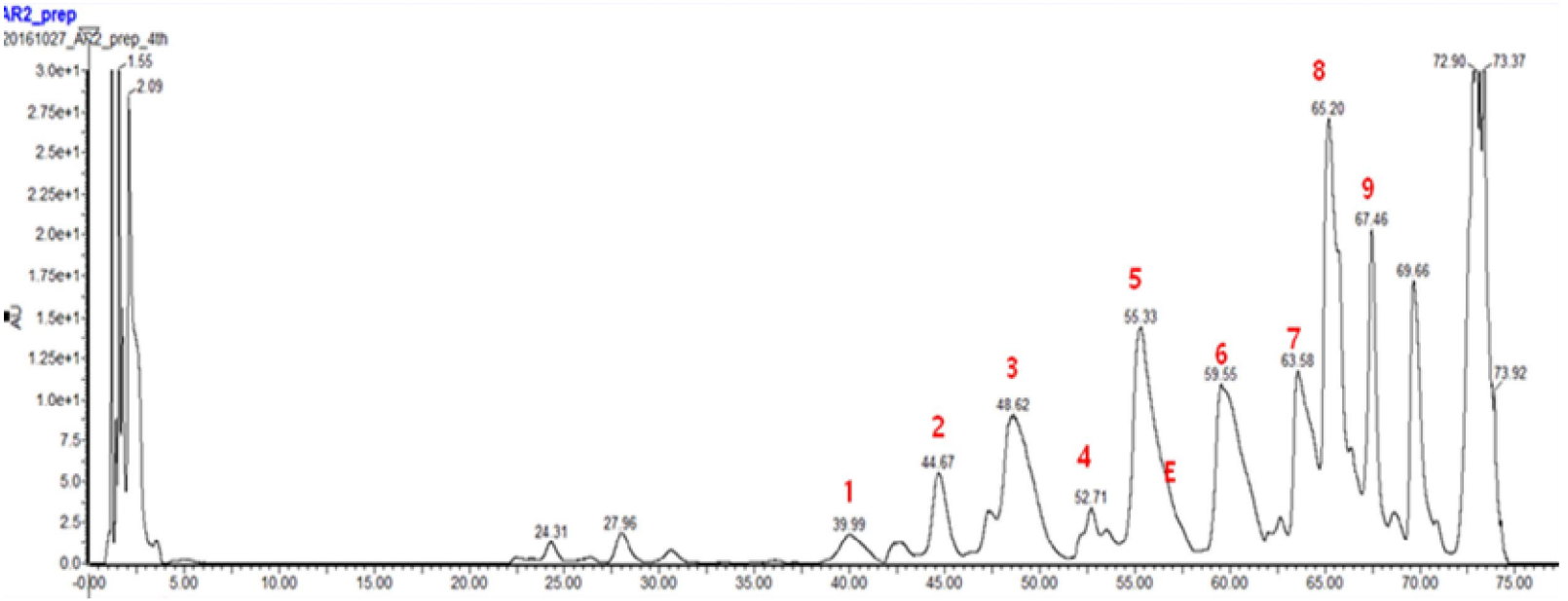
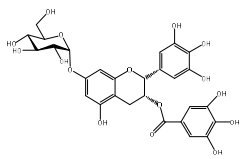
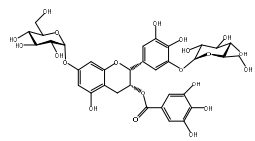
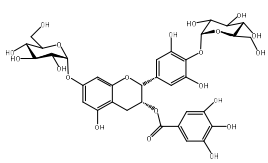
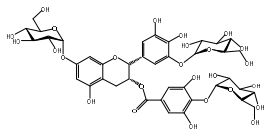
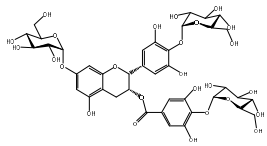
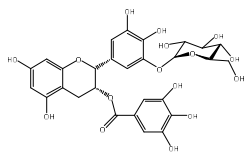
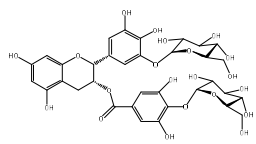
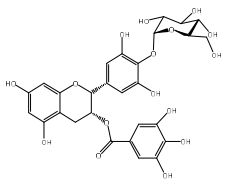
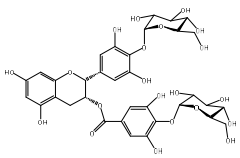


Figure1. Chromatogram of HPLC purification of acceptor reaction products using C18 prep column. 1~9 of EGCG-Gs were purified and EGCG was also purified (E).

Table 2. Quantity and structure of EGCG-Gs after purification

Compound	Name	mg	Structure	
			Existed	New
1	EGCG-7-O-α-D-glucopyranoside	6		
2	EGCG-7,5'-O-α-D-glucopyranoside	5.8		
3	EGCG-7,4'-O-α-D-glucopyranoside	9.5		
4	EGCG-7,5',4''-O-α-D-glucopyranoside	3.5		
5	EGCG-7,4',4''-O-α-D-glucopyranoside	8.5		
6	EGCG-5'-O-α-D-glucopyranoside	8		
7	EGCG-5',4''-O-α-D-glucopyranoside	5.5		

8	EGCG-4'-O-α-D-glucopyranoside	6.4		
9	EGCG-4',4''-O-α-D-glucopyranoside	3.9		

2. Effects of Glucosylation on Water Solubility.

We carried out a comparison of the water solubility of the EGCG and its glycosides. The solubility of 2, 4, 5, 6 and 7 were 754, 229, 1175, 1878 and 850 mM, whereas the solubility of EGCG was 5 mM. Their solubility was 139, 42, 217, 347 and 157 times as high as that of EGCG. Moon et al. [12] also reported that improving of water solubility of glycosylated EGCG (epigallocatechin gallate 7-O- α -D-glucopyranoside, epigallocatechin gallate 4' -O- α -D-glucopyranoside and epigallocatechin gallate 7,4' - O-α-D-glucopyranoside)by 49 to 117 times higher than EGCG. Improving of solubility by glycosilation of catechin is also studied (3' -O- α -D-glucopyranoside) by a factor of 50-fold, as compared to that of unglycosylated catechin [28]. Table 3 shows that the transference of a glucosyl residue to EGCG affected to an increase in the water solubility of the EGCG glycosides and compound 6 which is attached by one glucosyl residue to 5' of B ring has the highest water solubility.

Table 3. Water solubility of EGCG-g and relative solubility

Sample	Solubility in water(mM)	Relative solubility
EGCG	5.4	1
2(EGCG-7,5'-O-a-D-glucopyranoside)	754.56	139
4(EGCG-7,5',4''-O-a-D-glucopyranoside)	229.69	42
5(EGCG-7,4',4''-O-D-glucopyranoside)	1175.23	217
6(EGCG-5'-O-a-D-glucopyranoside)	1878.47	347
7(EGCG-5',4''-O-a-glucopyranoside)	850.85	157

3. Expression of human tyrosinase gene in *Escherichia coli.*

Human tyrosinase was expressed in *E.coli* after IPTG induction. To express larger amounts of human tyrosinase in *E. coli*BL21(DE3)pLysS, the optimal IPTG concentration and temperature for induction were determined. 1 mM IPTG was used to induce human tyrosinase at 16 ℃.

The yield of human tyrosinase was 0.5 mg/mL and the size of the enzyme was 64 kDa (Figure 2.)

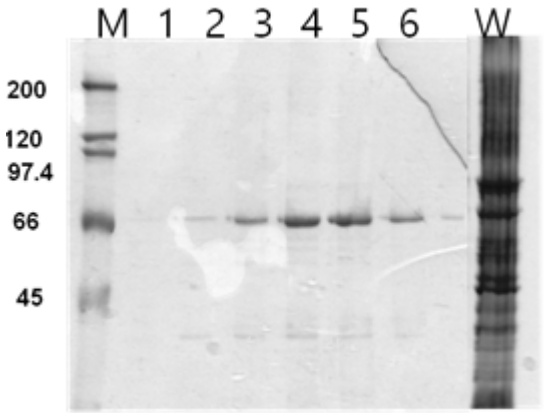


Figure 2. SDS-PAGE of purified HTY expressed in *E.coli* BL21(DE3). Lane M, the molecular mass marker; lane W, cell lysate with 0.5 mM IPTG induction to express HTY; lane1-6. Purified HTY after Ni-NTA column chromatography.

4. All EGCG glucosides enhanced human tyrosinase activity

Tyrosinase activation has not been studied in detail. However, some research of tyrosinase activation present several mechanisms. Shabani et al. [27] studied that fatty acids activated mushroom tyrosinase by conformational change of the enzyme by binding hydrophobic tail of fatty acids and effector site of the enzyme. In addition, catechin is also estimated that it changed tyrosinase protein and activated the enzyme by circular dichroism assay (CD) [29]. Tyrosinase is a kind of polyphenol oxidase (PPO). It means tyrosinase oxidase not only tyrosin but also polyphenol compounds. Catechin can be metabolized by tyrosinase to form o-quinone, which reacts with glutatihione (GSH) [30]. When o-quinone oxidased by tyrosinase conjugate with SH compounds such as cystein or GSH, melanin is synthesized. When catechin including EGCG exist with tyrosinase, it is used as substrates without L-DOPA or tyrosine [29, 31].

The table 4. shows EGCG-G activates human tyrosinase up to 70%. Glycosylated EGCG compounds activated human tyrosinase from low concentration (0.25 mM) on the other hand, EGCG activated the enzyme from 1.5 mM .

Extra experiment was conducted to verify that human tyrosinase is activated by using EGCG as substrate. Without L-DOPA used as substrate for tyrosinase, when only EGCG and human tyrosinase reacted to each other without L-DOPA, tyrosniase was activated using EGCG. Figure 3. shows the spevtrophotometric scanning of

the colored compounds resulting from the enzymatically-oxidized end products of catechin. This result shows that the tyrosinase-catechin biocatalysis gave distinct absorbency peak with maximum absorbance at 475 nm for the colored compounds of the enzymatically produced o-quinones. In addition, the result shows that in the absence of the EGCG in the reaction mixture, there was no indication of these peaks. These findings suggest that the bioconversion of EGCG into the corresponding o-quinones required the presence of tyrosinase. Figure 4. shows that more activation of human tyrosinase reacted with L-DOPA and EGCG together than with only L-DOPA. Additionally, EGCG conversion percentage is also calculated by TLC after 70 min (Figure 5., Tabe 5.)

Therefore, EGCG plays a role of substrate to human tyrosinase and then affects to quinone production which synthesize melanin.

Table 4. The effects of EGCG and EGCG glycosides on biological activity of tyrosinase.

Compound	Concentration (mM)	Activation (%)
EGCG	0.25	71.58
	0.5	72.98
	1	69.08
	1.5	119.35
	2	135.37
1	0.25	96.91±6.4
	0.5	103.52±2.0
	1	111.83±16.2
	1.5	98.70±2.34
	2	107.25±8.7
2	0.25	109.12±2.7
	0.5	112.67±1.9
	1	121.43±0.7
	1.5	122.91±5.9
	2	140.45±14.3
3	0.25	105.24±2.4
	0.5	109.42±1.8
	1	179.72±5.9
	1.5	130.63±6.2
	2	128.73±18.8
4	0.25	110.15±3.0
	0.5	113.08±10.7
	1	118.00±2.8
	1.5	118.94±1.9
	2	123.14±2.7

5	0.25	103.38±2.9
	0.5	112.33±6.1
	1	107.11±1.8
	1.5	111.83±1.4
	2	131.92±31.4
6	0.25	123.17±4.1
	0.5	125.26±9.3
	1	129.65±13.1
	1.5	135.36±2.9
	2	147.58±16.9
7	0.25	117.46±2.9
	0.5	120.20±8.4
	1	127.84±8.7
	1.5	137.67±5.2
	2	150.07±9.8
8	0.25	106.40±4.2
	0.5	114.75±6.2
	1	128.79±6.3
	1.5	120.94±5.7
	2	115.12±8.1
9	0.25	113.24±0.9
	0.5	122.51±7.3
	1	141.14±21.1
	1.5	129.27±17.6
	2	119.78±14.3

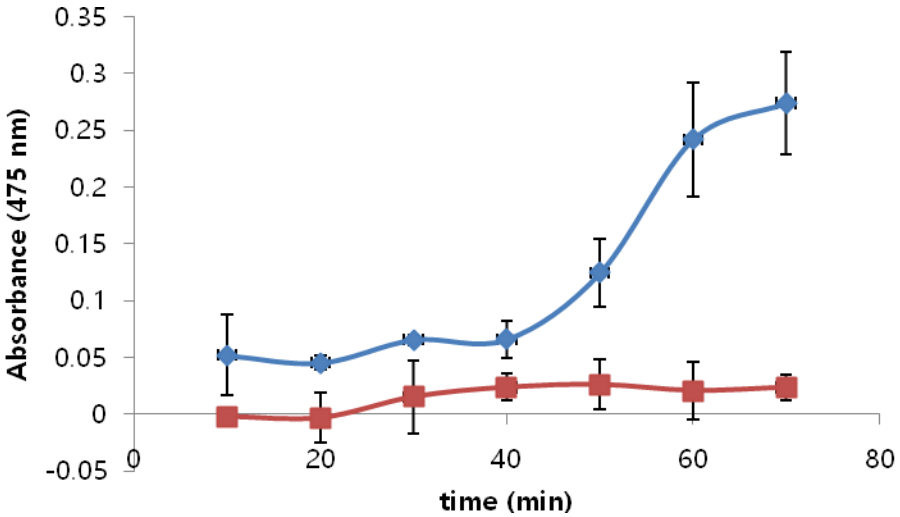


Figure 3. Human tyrosinase activity when reacted with EGCG and without any substrate. 2 mM of EGCG and human tyrosinase(●), human tyrosinase without any substrate (■).

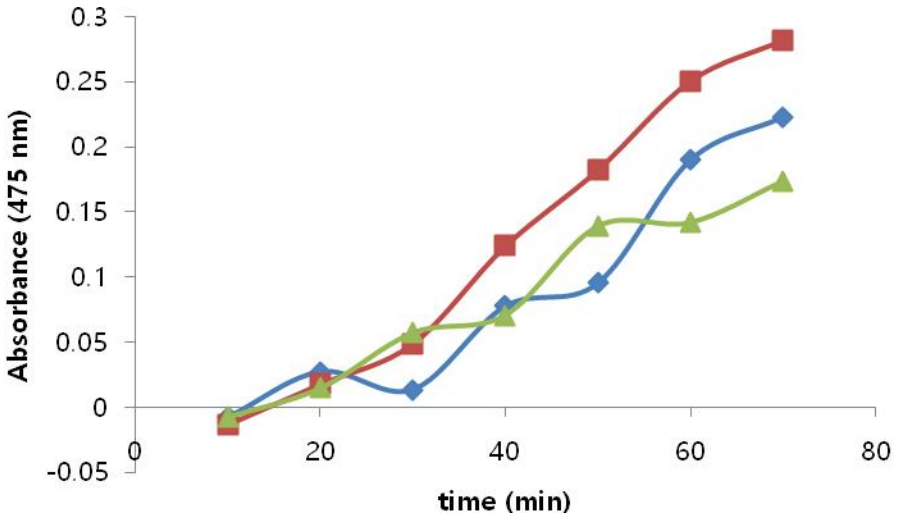


Figure 4. Human tyrosinase activity in differnet condition of substrate. 2 mM of EGCG and human tyrosinase (●), 2 mM EGCG , 3.3 mM of L-DOPA and human tyrosinase (■), 3.3 mM of L-DOPA and human tyrosinase (▲).

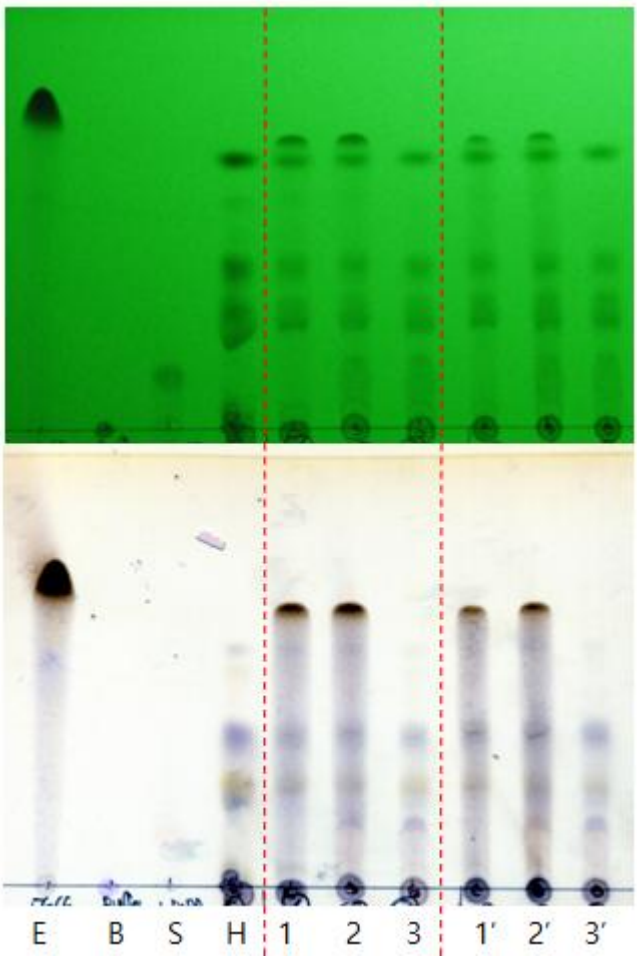


Figure 5. Thin layer chromatogram of reaction mixture in different condition of substrate: E, 50 mM EGCG; B, 450 mM Ptassium phosphate buffer; S, 10 mM L-DOPA; 1, 2 mM EGCG and human tyrosinase (0 min); 2, 2 mM EGCG, 3.3 mM L-DOPA and human tyrosinase (0 min); 3, 3.3 mM L-DOPA and human tyrosinase; 1', 2', 3', after 70 min.

Table 5. EGCG conversion rate after 70 min.

	EGCG conversion after 70 min (%)
1	34.4
2	23.5

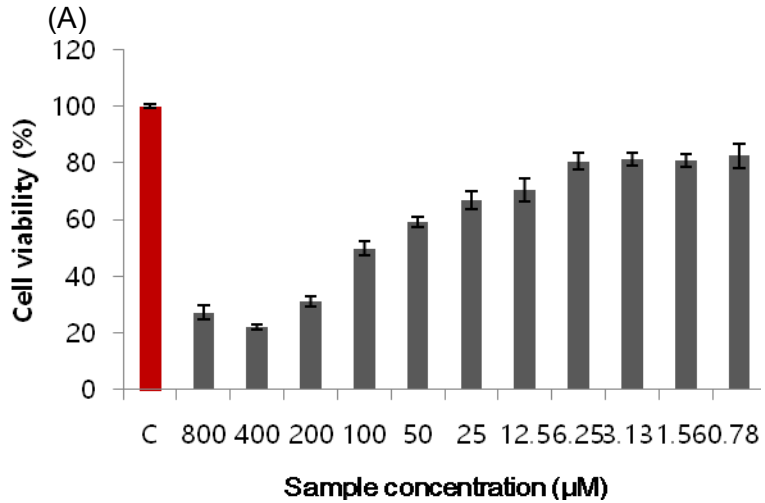
1, 2 mM EGCG and human tyrosinase; 2, 2 mM EGCG, 3.3 mM L-DOPA and human tyrosinase.

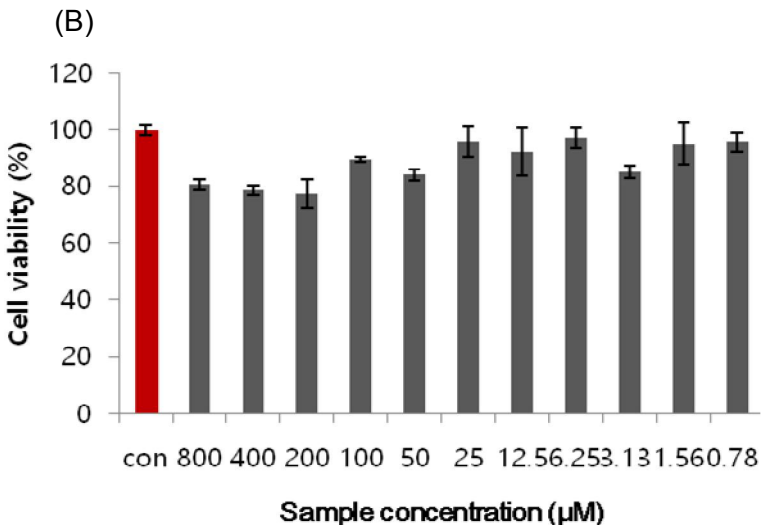
5. Cell viability

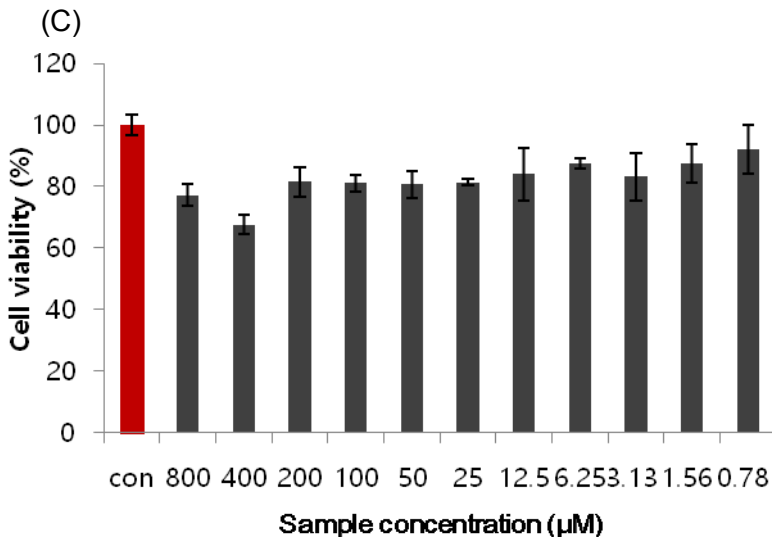
To examine whether EGCG glycosides have cytotoxicity effects, we treated Raw cells and B16F10 melanoma cells with this compounds at various concentrations; cell viability was determined by using WST assay. There are several reports found that EGCG arrested growth of lung, colon and ovarian cancer cells [32-34]. Polyphenol including EGCG treatment decreased cell viability in human leukemia cell line K562 and the human embryoinic kidney 293T cell line [35].

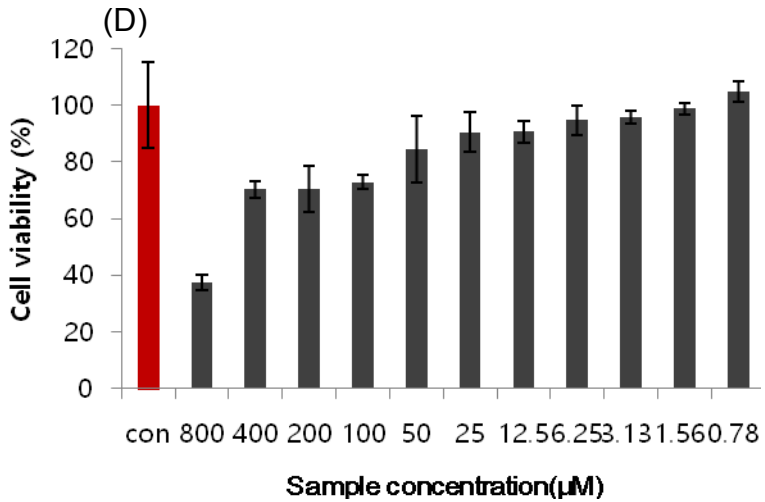
Especially, EGCG showed the strongest inhibition of cell proliferation (approximately, 22% at 400 μM, 70% at 12.3 μM to Raw cells and 38% at 400 μM, 75% at 50 μM to B16F10 cells) and some cells detached from the plates. Overall EGCG glycosides did not inhibit cell proliferation of both Raw cells(Figure 6.) and B16F10 melanoma cells (Figure 7.) at high concentration comparatively with EGCG. Compound 7 is supposed that it doesn't have cell cytotoxicity to Raw cells and B16F10 cells at high concentration. Compound 2 and 3 also showed no inhibition of B16F10 cells proliferation at full concentration (viability was more than 80%). Therefore the maximum treatment concentration of glucosylated EGCG samples was higher than it of EGCG. In Raw cells, treatment concentration of EGCG was 6.25 μM, compound 2 was 50 μM, 4 was 100 μM, 5 was 50 μM, 6 was 25 μM and 7 was 200 μM (Table 6.). In B16F10 cells, treatment concentration of EGCG was 25 μM, 2 was 50 μM, 4 was 400 μM, 5 was 200 μM, 6 was 25 μM and 7 was 400 μM (Table 7.). Compound 6 has only one glucosyl residue at 5' of B ring and its cell cytotoxicity

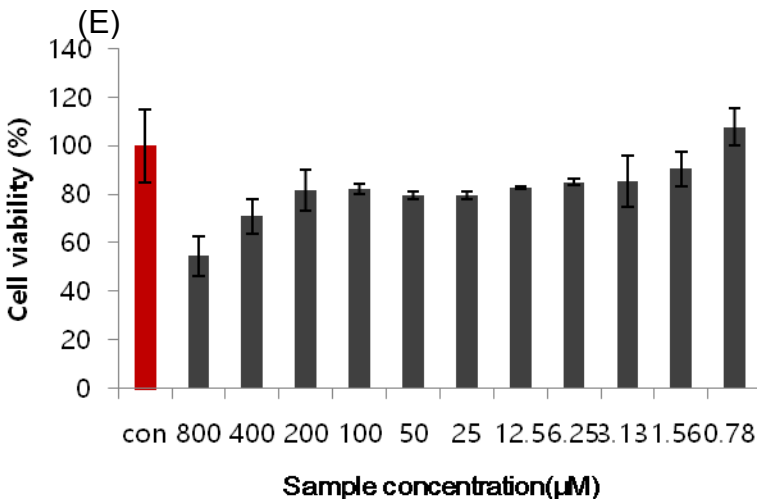
was higher than other compounds which have glucosyl residues more than two. It means that the number of attached glucosyl residues plays a clear role in cell viability. We supposed that the more glucosyl residues attached to EGCG, the weaker cell cytotoxicity.


(A)
Cell viability (%)
120
100
80
60
40
20
0
C 800 400 200 100 50 25 12.5 6.25 3.13 1.56 0.78
Sample concentration (μM)


(B)
Cell viability (%)
120
100
80
60
40
20
0
con 800 400 200 100 50 25 12.5 6.25 3.13 1.56 0.78
Sample concentration (μM)


(C)
Cell viability (%)
120
100
80
60
40
20
0
con 800 400 200 100 50 25 12.5 6.25 3.13 1.56 0.78
Sample concentration (μM)


(D)
Cell viability (%)
120
100
80
60
40
20
0
con 800 400 200 100 50 25 12.56.253.131.560.78
Sample concentration(μM)


(E)
Cell viability (%)
140
120
100
80
60
40
20
0
con 800 400 200 100 50 25 12.56.253.131.560.78
Sample concentration(μM)

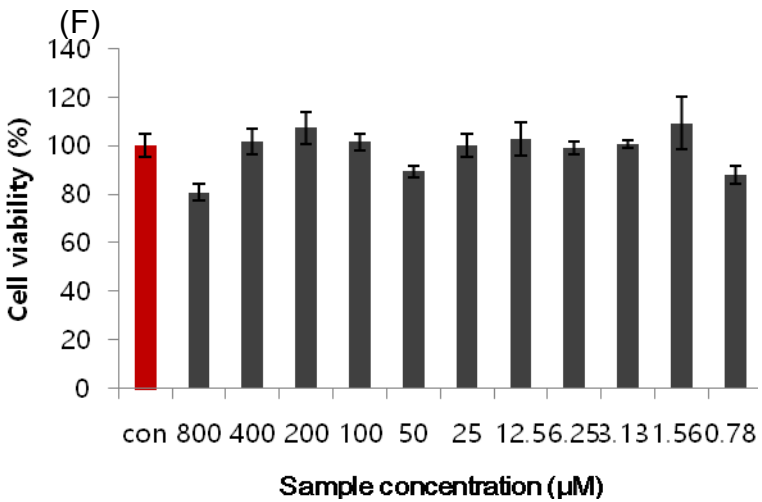
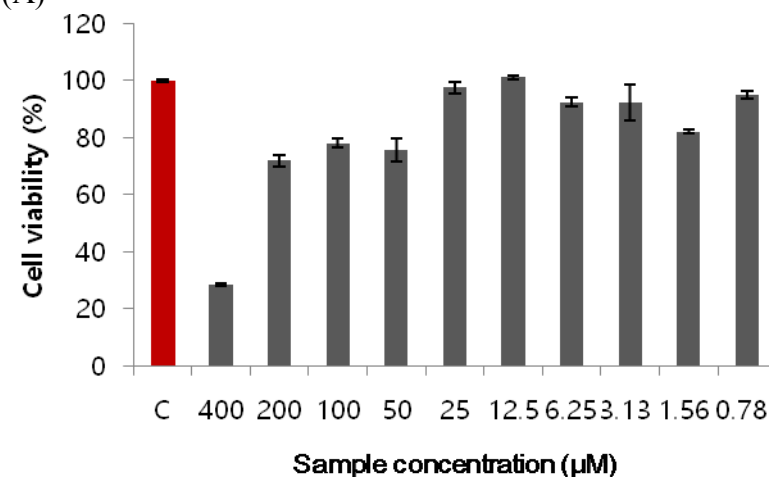

(F)
Cell viability (%)
140
120
100
80
60
40
20
0
con 800 400 200 100 50 25 12.56.253.131.560.78
Sample concentration (μM)

Figure 6. Effect of EGCG and EGCG glycosides on cell viability in cultured Raw cells. Data are expressed as percent change of the cell viability level relative to untreated control. Each determination was made in triplicate.
(A) EGCG, (B) 2, (C) 4, (D) 5, (E) 6, (F) 7

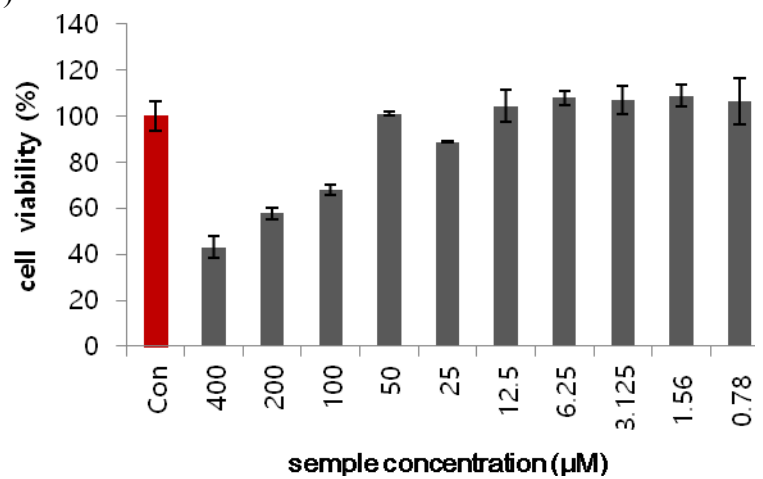
Table 6. Maximum concentration of EGCG-g to treat to Raw 264.7 cells

Cytotoxity assay	Treatment concentration(μM)
EGCG	6.25
2(EGCG-7,5'-*O*-α-D-glucopyranoside)	50
4(EGCG-7,5',4''-*O*-α-D-glucopyranoside)	100
5(EGCG-7,4',4''-*O*-α-D-glucopyranoside)	50
6(EGCG-5'-*O*-α-D-glucopyranoside)	25
7(EGCG-5',4''-*O*-α-D-glucopyranoside)	200

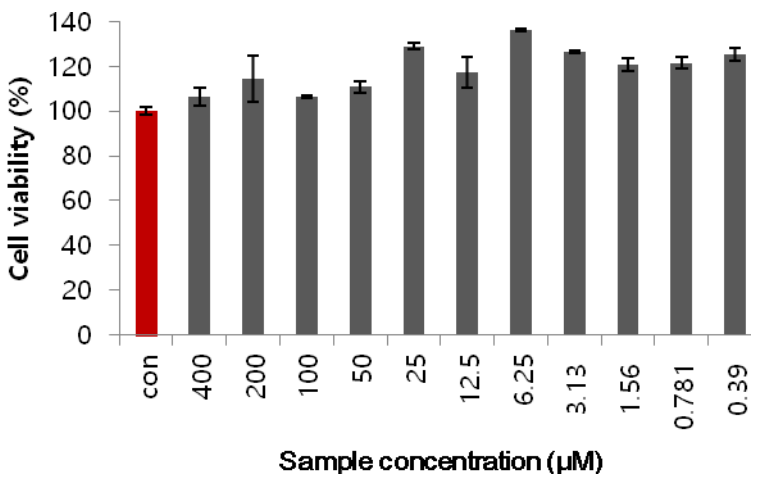
(A)


120
100
80
60
40
20
0
Cell viability (%)
C 400 200 100 50 25 12.5 6.25 3.13 1.56 0.78
Sample concentration (μM)

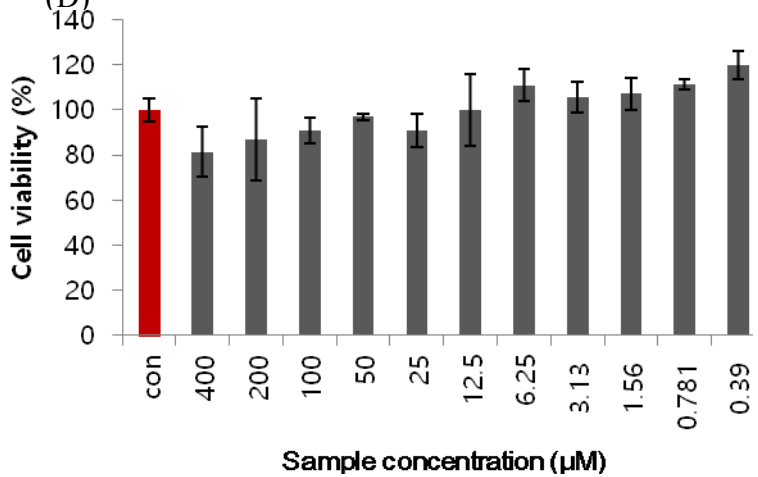
(B)


140
120
100
80
60
40
20
0
cell viability (%)
Con 400 200 100 50 25 12.5 6.25 3.125 1.56 0.78
semple concentration (μM)

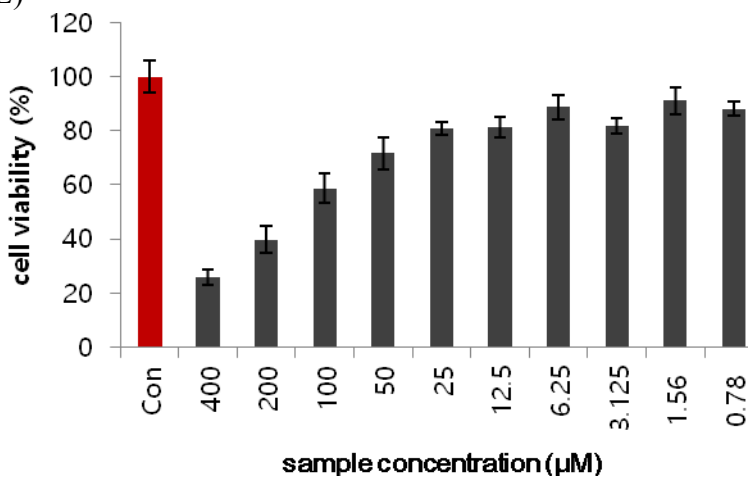
(C)


140
120
100
80
60
40
20
0
Cell viability (%)
con 400 200 100 50 25 12.5 6.25 3.13 1.56 0.781 0.39
Sample concentration (μM)

(D)


Cell viability (%)
140
120
100
80
60
40
20
0
con
400
200
100
50
25
12.5
6.25
3.13
1.56
0.781
0.39
Sample concentration (μM)

(E)


cell viability (%)
120
100
80
60
40
20
0
Con
400
200
100
50
25
12.5
6.25
3.125
1.56
0.78
sample concentration (μM)

(F)

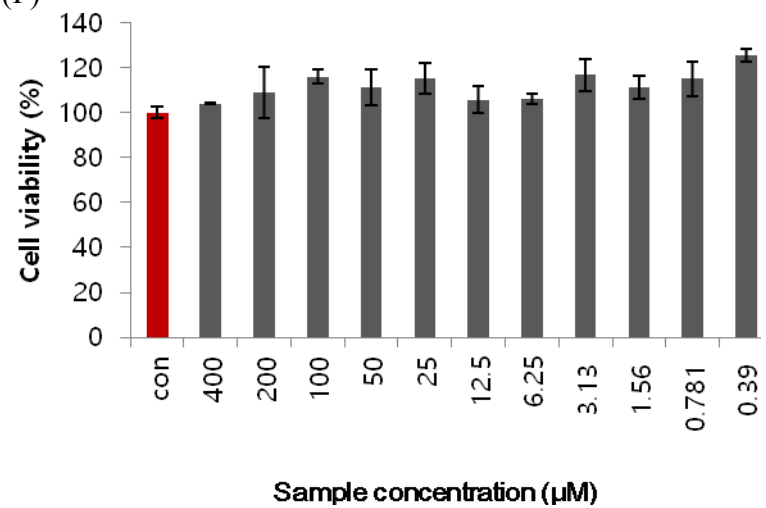

Cell viability (%)
140
120
100
80
60
40
20
0
con
400
200
100
50
25
12.5
6.25
3.13
1.56
0.781
0.39
Sample concentration (μM)

Figure 7. Effect of EGCG and EGCG glycosides on cell viability in cultured B16F10 melnoma cells. Data are expressed as percent change of the cell viability level relative to untreated control. Each determination was made in triplicate.
(A) EGCG, (B) 2, (C) , (D) 5, (E) 6, (F) 7

Table 7. Maximum concentration of EGCG-g to treat to B16F10 cell

Cytotoxity assay	Treatment concentration(μM)
EGCG	25
2(EGCG-7,5'-*O*-α-D-glucopyranoside)	50
4(EGCG-7,5',4"-*O*-α-D-glucopyranoside)	400
5(EGCG-7,4',4"-*O*-α-D-glucopyranoside)	200
6(EGCG-5'-*O*-α-D-glucopyranoside)	25
7(EGCG-5',4"-*O*-α-D-glucopyranoside)	400

6. Effect of EGCG and EGCG-Gs on melanin content in B16F10 cells

To examine the melanin synthesis in the epidermal cell, EGCG and EGCG glucosides were treated on B16F10 melanoma cells. α-MSH stimulated the melanin synthesis and α-arbutin, β-arbutin and kojic acid inhibit the melanin metabolism as postive controls.

500 nM of α-MSH increased melanin contents up to 60%. When the same concentration of EGCG and EGCG glucosides was treated, the most effective in inibition of melanin synthesis was compound 4 (EGCG-7,5',4''-O-α-D-glucopyranoside). EGCG glucosides have similar effect of skin bruightening effect with EGCG.

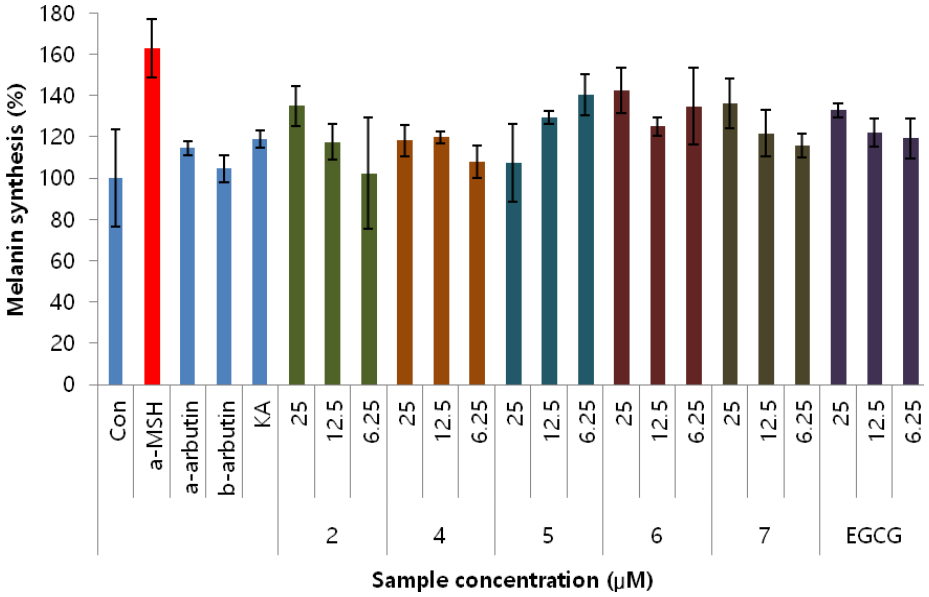


Figure 8. Effect of EGCG and EGCG-Gs on melanin content in B16F10 cells All data are presented as mean±standard deviation.

7. Measurement of NO production

Treatment of LPS is known for stimulation of related factors such as NO and TNF-α by inflammatory reaction. Overexpressed NO or TNF-α have negative effects to human body causing systemic inflammation. However proper amount of NO is considered as a key factor of congenital immunity. In this study, we conducted quantification analysis of NO production by treatment of EGCG and EGCG glycosides to LPS treatment group.

LPS treatment group significantly produced NO more than 34% compared to untreated control group. We confirmed that indomethacine suppressed production of NO increased by LPS stimulation as a positive control. EGCG and EGCG glycosides inhibit secretion of NO form Raw cells up to 68% and especially, compound 7 has the strongest inhibition ability concentration dependently. Treatment concentration of compound 7 was higher than others because of its low cell cytotoxicity (Figure 9.).

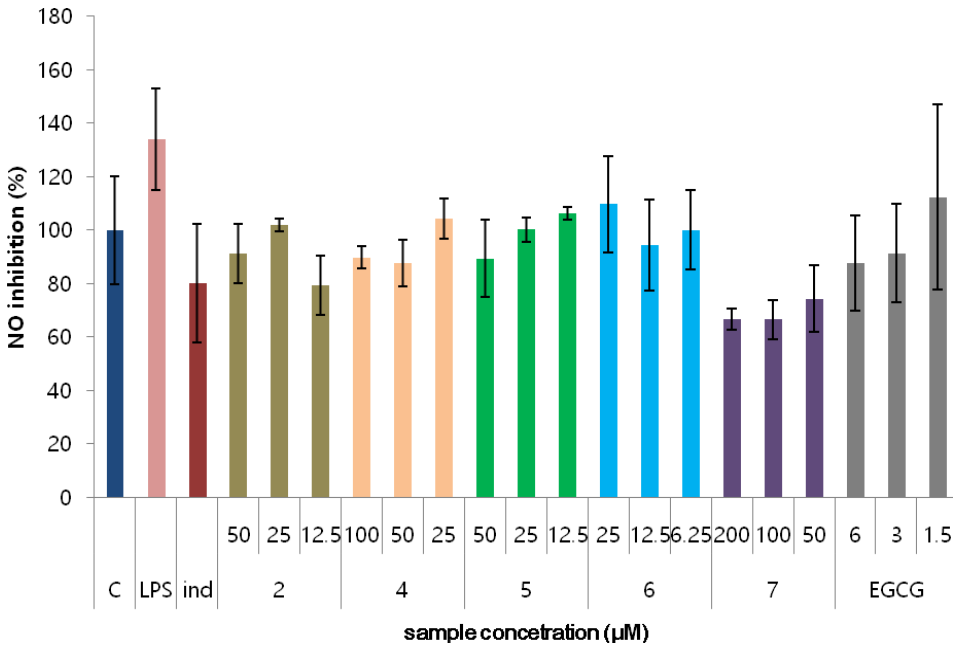


Figure 9. Effects of EGCG and EGCG glycosides on the nitric oxide production by Raw 264.7 cells. All data are presented as mean±standard deviation. Data are expressed as percent change of the nitric oxide production level relative to untreated control. Each determination was made in triplicate.

Conclusion

Though EGCG plays an important role in various biofunction, it is difficult to apply to cosmetic, pharmaceutical and food industry because of its low solubility. We solve the problem by acceptor reaction to make the glucosidic bond to EGCG and developed novel EGCG glucosides which is not exist.

Novel compounds which have glucosidic bond at new position were purified and these are much higher-water soluble than EGCG water solubility. Especially, compound 6 (EGCG-5'-O-α-D-glucopyranoside) has 348 -folds higher solubility than EGCG aglycon.

Human tyrosinase was expressed in *E.coli* and EGCG and its glucosides activate the enzyme unlike mushroom tyrosinase. We confirmed that EGCG and EGCG-Gs act as substrate like L-DOPA.

In biochemical properties, EGCG-Gs have less cell cytotoxicity on epidermal cells. Therefore it is possible to treat at higher concentration on Raw 264.7 cells and B16F10 melanoma cells to prevent aging skin damaged from UV.

EGCG -Gs have more inhibition ability on production of nitric oxide on Raw 264.7 cells. The compound 7 (EGCG-5',4''-*O*-α-D-glucopyranoside)with the lowest cell cytotoxicity on epidermal cells inhibit the production of nitric oxide up to 68%. In addition, the compound 4 (EGCG-7,5',4''-O-α-D-glucopyranoside) with three glucosidic bond inhibit the melanin synthesis 40%.

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easing the activity and synthesis of tyrosinase in human mela noma cells and in normal human melanocytes. Pigment cell r esearch, 2004. **17**(1): p. 66-73.
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국문 초록

녹차에 가장 풍부하게 존재하는 폴리 페놀 물질인 에피갈로카테킨 갈레이트 (EGCG)는 항암, 항산화, 체중 감량 등의 효과가 있다. 그러나 다양한 이점에도 불구하고 물에 대한 용해도가 낮아 여러 산업에서 이용하기에 제한이 있다. 본 연구에서는 대장균에서 발현된 *Leuconostoc mesenteroides* B-1299CB에서 생산한 덱스트란 수크레이즈를 이용하여 EGCG배당체를 만들고 HPLC C_{18} 컬럼으로 그들의 증가된 수용성을 확인하였다. 새로이 발견한 다섯 개의 배당체 물질들은 42-347배까지 수용성이 증가하였고, 특히 6번 배당체 (EGCG-5'-O-a-D-glucopyranoside)가 1878.47 mM로 가장 수용성이 높았다. 또한 EGCG는 버섯 타이로시네이즈에 저해능이 있어 미백효과가 있다고 연구되어 왔지만 본 연구에서 직접 인간 타이로시네이즈를 발현하여 실험한 결과 그와 반대로 EGCG 및 EGCG 배당체가 인간 타이로시네이즈의 기질로서 사용이 된다는 것을 확인하였다. 생화학적 특징으로는 배당체 물질이 피부세포에 미치는 세포 독성이 현저히 줄었으며, Raw264.7 세포에서 생성하는 NO 생성량을 최대 68%까지 감소시켰다. 또한 B16F10 세포의 멜라닌 생성 억제능 확인에서도 4번 배당체 (EGCG-7,5',4''-O-α-D-glucopyranoside)가 최대 40%까지 멜라닌 생성을 억제하였다.

주요어: 에피갈로카테킨 갈레이트, 수용체 반응, 인간 타이로시네이즈, 멜라닌, NO 억제능

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