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# Glycosylation and subsequent malonylation of isoflavonoids in *E. coli*: strain development, production and insights into future metabolic perspectives

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**Abstract** Genistin and daidzein exhibit a protective effect on DNA damage and inhibit cell proliferation. Glycosylation and malonylation of the compounds increase water solubility and stability. Constructed pET15b-GmIF7GT and pET28a-GmIF7MAT were used for the transformation of *Escherichia coli* and bioconversion of genistein and daidzein. To increase the availability of malonyl-CoA, a critical precursor of GmIF7MAT, genes for the acyl-CoA carboxylase α and β subunits (*nfa*9890 and *nfa*9940), biotin ligase (*nfa*9950), and acetyl-CoA synthetase (*nfa*3550) from *Nocardia farcinia* were also introduced. Thus, the isoflavonoids were glycosylated at position 7 by 7-*O*-glycosyltranferase and were further malonylated at position 6″ of glucose by malonyl-CoA: isoflavone 7-*O*-glucoside-6″-*O*-malonyltransferase both from *Glycine max*. Engineered *E. coli* produced 175.7 μM (75.90 mg/L) of genistin and 14.2 μM (7.37 mg/L) genistin 6″-*O*-malonate. Similar conditions produced 162.2 μM (67.65 mg/L) daidzin and 12.4 μM (6.23 mg/L) daidzin 6″-*O*-malonate when 200 μM of each substrate was supplemented in the culture. Based on our findings, we speculate that isoflavonoids and their glycosides may prove useful as anticancer drugs with added advantage of increased solubility, stability and bioavailability.

**Keywords** Glycosylation · Malonylation · Bioconversion · Isoflavonoids · Anticancer

## Introduction

Isoflavonoids are a large subclass of flavonoids having a 15-carbon (C6–C3–C6) backbone arranged as a 1,2-diphenylpropane skeleton. There are over 1,000 structures of isoflavonoids and their derivatives and over 5,000 structures of flavonoids in total [25]. These isoflavonoids are biologically active secondary metabolites produced by most of the leguminous plants and have been reported to be produced by non-leguminous plants as well. Isoflavonoids are derived from the basic 3-phenylchroman backbones of isoflavones by various modifications, such as methylation, hydroxylation, or polymerization. These modifications lead to simple isoflavonoids such as isoflavanones, isoflavans, and isoflavonols as well as more complex structures such as rotenoids, pterocarpans, and coumestans [5].

The physiological function of isoflavones in humans and animals continues to be the subject of intense investigation. Research has found that these polyphenolic compounds may reduce the occurrences of certain types of cancer (example: breast cancer, prostate cancer, and colon cancer), reduce postmenopausal symptoms, prevent coronary heart disease by reducing low-density lipoprotein and increasing high-density lipoprotein, and have positive effects on other physiological processes such as neurogenesis and brain functions [4, 19, 28, 34, 35].

In pharmaceutical studies, it was found that glycosylated flavonoids possessing biological activities, stability and solubility, are more efficacious than their parent molecules [12, 20]. Although all isoflavones are efficiently absorbed from the intestinal tract, there are striking difference in the

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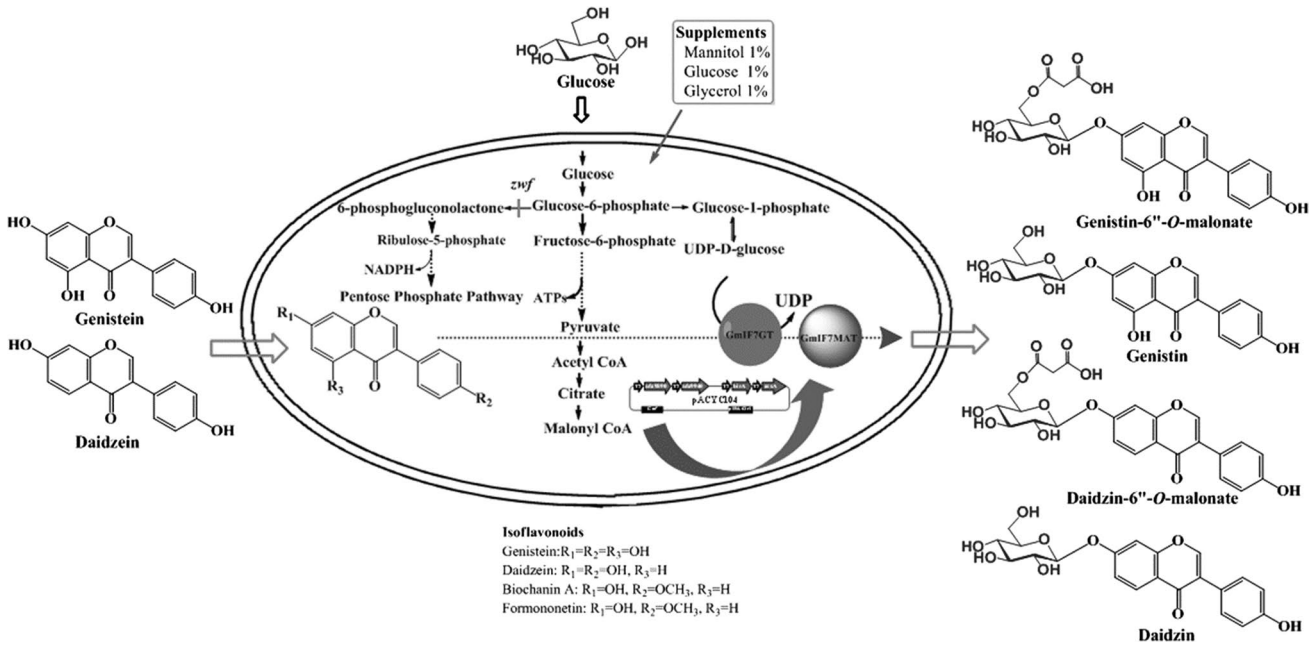
fate of aglycones and β-glucosides. After ingestion, the glucoside forms are hydrolyzed, mainly by β-glucosidase of the intestinal microflora, into aglycone forms that are readily absorbed through the intestinal enterocyte [30]. It was proven that glucosides genestin (genistein-7-*O*-β-D-glucoside) and daidzin (daidzein-7-*O*-β-D-glucoside) were partly absorbed from the intestine without previous cleavage and do not require hydrolysis to be biologically active [1]. Genistin also arrests growth of human melanoma cells in vitro and inhibits UV-light-induced oxidative DNA damage [27, 36]. It was found that both genistin and daidzin exhibited a protective effect on DNA damage and exhibited a superoxide dismutase-like effect, but only genistin significantly reduced the vitality of human melanoma cell lines, confirming the importance of the 5,7-dihydroxy structure in the A ring [1]. Genistein and genistin inhibit cell proliferation by disrupting the cell cycle, which is strongly associated with the arrest induction of either the G1 or the G2/M phase and may induce apotopsis [3]. In addition, a preparation of a herb rich in isoflavone glucoside, such as genistin and daidzin from soya, stimulated the production of hyaluronic acid in normal human epidermal keratinocytes and thus could be used as a new cosmetic ingredient in moisturizers and anti-aging agents [37].

Glucose moieties of phenolic glucosides, such as flavonoid-glucosides, are often modified by further glycosylation or acylation in plants [38]. The proposed roles of malonylation in plants include the stabilization of labile structures, the enhancement of the solubility of target compounds in water, and the transport of target compounds into the vacuole [8, 18, 31]. The malonylated flavonoid glucosides were more efficiently taken up into multidrug and toxin extrusion transporter (MATE2) containing vesicles than the parent glycosides were. Malonylation increases both the affinity and transport efficiency of flavonoid glucosides for uptake by MATE2 [39]. Genistin 6″-*O*-malonate and daidzin 6″-*O*-malonate exhibit remarkable antioxidant properties, being capable of protecting fats, vitamins, and/or oligoelements present in cosmetics or food products against oxidation [7].

Naturally extracted isoflavonoid glucosides and malonylated isoflavonoid glucosides from plants can be marketed as commercial goods, but the high cost of production is a major obstacle which limits availability. With the advances in metabolic engineering, there are various approaches to enhance the product yield of glycosylated flavonoids in *E. coli*, including gene deletion, over-expression of sugar gene cassettes, protein engineering, biotransformation increment of the substrate, as well as improvement of product excretion to the outside of the cell [10, 14]. Here, we report a metabolic engineering approach for conjugating a malonyl and glucose moiety generated in the cytoplasm of *E. coli* with exogenously supplied genistein and daidzein by whole cell biotransformation (Scheme 1). As the efficiency of triple mutants in increasing the UDP-glucose pool inside the cell of *E. coli* was already proven [23], we used the same mutant for the biotransformation using a fermentor. Furthermore, *galU* was proven to




**Scheme 1** Generalized scheme for the production of glycosylated isoflavonoids and derivatives in genetically engineered *E. coli.* 1 % glucose, 1 % mannitol and 1 % glycerine was supplemented for the large scale production by fermentation

amplify UDP-glucose in *Lactobacillus casei* or engineered *E. coli* [26]. Additionally, to increase the intracellular malonyl-CoA pool in *E. coli*, we redesigned the acetate-assimilating and malonyl-CoA synthetic pathways using the enzymes from *Nocardia farcinica*. Furthermore, water solubility of isoflavonoids and their derivatives was compared and both anti-cancer and anti-angiogenesis activity were assayed in vitro.

## Materials and methods

### DNA, plasmids and bacterial strains

Recombinant DNA techniques were performed according to standard pocedures [29]. Restriction enzymes, *LaTaq* polymerase, and T4 DNA ligase were purchased from Takara Bio (Shiga, Japan) and Promega (Madison, WI, USA). Oligonucleotide primers were synthesized from Genotech (Daejeon, Korea). Details of all primers used are available from the authors on request.

pET-15b-GmIF7GT [21], pQE30-GmIF7MaT [32] and pACYC-104 [17] plasmids were confirmed by restriction digestion and PCR and used for transformation. The expression of the transformed plasmids was checked by SDS-PAGE. Three genes involved in the production of precursors of NDP-sugar in *E. coli* BL21 (DE3) viz. D-glucose phosphate isomerase (*pgi*), D-glucose-6-phosphate dehydrogenase (*zwf*), and UDP-sugar hydrolase (*ushA*) were deleted for the purpose of higher accumulation of intracellular UDP-glucose. The gene knock out was followed as described in NanoBio [9].

Finally, all constructed plasmids were transformed into the *E. coli* BL21 (DE3) and mutant *E. coli* BL21 (DE3) Δ*zwf* and *E. coli* BL21 (DE3) Δ*pgi*/Δ*zwf*/Δ*ushA* to generate *E. coli* M1, *E. coli* M2, *E. coli* M2G, *E. coli* M2GG, *E. coli* M1GM, and *E. coli* M1GMM. All vectors, plasmids, and strains used in this research are listed in Table 1.

### Culture media and reagents

The seed culture was grown in Luria–Bertani (LB) broth (Bacto, Sparks, MD, USA), Terrific broth (TB) (Bacto, Sparks, MD, USA), and M9 minimal media (per liter, 6 g $Na_2HPO_4$, 3 g $K_2HPO_4$, 0.5 g NaCl, 0.1 % $NH_4Cl$, 1 % glucose, 1 mM $MgSO_4.7H_2O$, 100 μM $CaCl_2$) supplemented with either kanamycin (50 μg/mL), ampicillin (50 μg/mL), or chloramphenicol (50 μg/mL) when necessary and was utilized for biotransformation of the substrates. In case of mutant strains, the culture broth was supplemented with

**Table 1** List of strains and plasmids used in this study

| Strains/plasmids | Description | Abbreviation | Source/reference |
|---|---|---|---|
| *E. coli* strains | | | |
| *Escherichia coli* BL21 (DE3) | Omp Thsd ThsdS (rB⁻mB⁻) gal(DE3) | | Novagen, Germany |
| *E. coli* XL-1 Blue (MRF) | General host far cloning | | Promega, WI, USA |
| *E. coli* BL21 (DE3)/Δzwf | *E coli* BL21 (DE3); zwf deleted | *E. coli* Ml | [23] |
| *E. coli* BL21 (DE3)/Δpgi/Δzwf/ΔushA | *E coli* BL21 (DE3); pgi/zwf/ushA deleted | *E. coli* M2 | [23] |
| *E. coli* BL21 (DE3)/Δpgi/Δzwf/ΔushA/galU | *E. coli* M2 carrying pET28a-galU | *E. coli* M2G | This study |
| *E. coli* BL21 (DE3)/Δpgi/Δzwf/ΔushA/galU/GmIF7GT | *E. coli* M2G carrying pET-l5b-GmIF7GT | *E. coli* M2GG | This study |
| *E. coli* BL21 (DE3)/Δzwf/GmIF7GT/GmIF7MaT | *E. coli* M1 carrying pET-15b-GmIF7GT and pET-28a-GmIF7MaT | *E. coli* M1GM | This study |
| *E. coli* BL21 (DE3)/Δzwf/GmIF7GT/GmIF7MaT/PACYC104 | *E. coli* M1GM carrying pACYC104 | *E. coli* M1GMM | This study |
| Plasmids vectors | | | |
| pET28a (+) | Expression vector | | Novagen, Germany |
| pET15b (+) | Expression vector | | Novagen, Germany |
| pACYCDuet-1 | Expression vector having double T7 promoters | | Novagen, Germany |
| pET28a-gaIU | pET28a carrying glucose-1-phosphate uridyltransferase from *E coli* K12 | | This study |
| pET-15b-GmIF7GT | pET-15b carrying Isoflavone 7-*O*-glucosyltransferase from *Glycine max* | | [21] |
| pET-28a-GmIF7MaT | pET-28a carrying Isoflavone 7-*O*-glucoside-6″-*O*-malonyltransferase from *Glycine max* | | This study |
| pACYC104 | pACYCDuetl carrying nfa9890, T7-rbs-nfa9940, nfa9950, and T7-rbs-nfa3550 from *N. fatcinic* | | [17] |

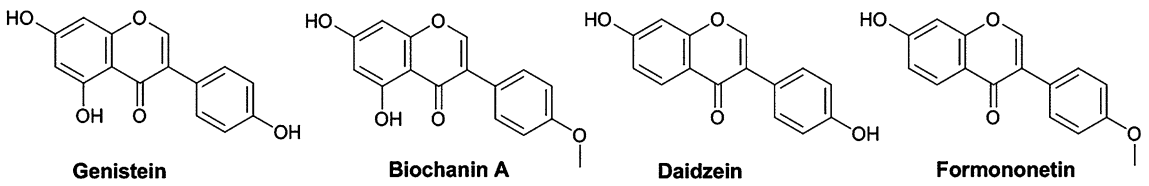



**Fig. 1** Structures of different isoflavonoids (genistein, biochanin A, daidzein and formononetin) used in this study

1 % mannitol, 1 % glucose, and 1 % glycerol. Authentic genistein, daidzein, formononetin and biochanin A (Fig. 1) were purchased from Tokyo Chemical Industry (Tokyo, Japan). All other chemicals were of high-grade quality and were purchased from commercial sources.

## Biotransformation method

We used *E. coli* BL21 (DE3) and mutant strains over expressing pET-15b-GmIF7GT, pET-28a-GmIF7MaT, and pACYC-104 plasmids for biotransformation of isoflavonoids. Culture inoculum was prepared in 3 mL LB liquid media with appropriate antibiotics and incubated at 37 °C with 220 rpm overnight. The following day 200 µL of pre-inoculum was transferred into 50 mL of LB liquid medium with antibiotic and cultured at 37 °C until the optical density at 600 nm ($OD_{600nm}$) reached approximately 0.6. Expression of the heterologous pathway was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to the final concentration of 0.5 mM after that and the cultures were incubated at 20 °C for 3 h. All substrates were dissolved in dimethyl sulfoxide (DMSO) and were brought to a final concentration of 200 µM. Then, incubation of the cultures was continued at 20 °C for 60 h in a shaking incubator operating at 220 rpm. The concentration of substrates and media composition were varied for production optimization by biotransformation. Every 12 h for 60 h, 500 µL of culture was collected and extracted by double a volume of ethyl acetate. The organic layer was collected and concentrated to dryness by evaporation of excess solvent. The remaining products were dissolved in 500 µL of methanol for chromatographic analyses.

## Extraction, purification, identification and quantification of metabolites

In brief, the crude extract was obtained by extracting culture broth with double volume of ethyl acetate (*v*/*v* = 2:1), stirred, and dried by frozen rotary evaporator. The elimination of the fat content was done by addition of hexane and methanol (*v*/*v* = 1:5). The crude extract was further purified by preparative HPLC. The productions of glycosylated isoflavonoids and their malonates in *E. coli* recombinants were analyzed and quantified by high-performance liquid chromatography coupled with photo diode array (HPLC–PDA) (Shimadzu, Japan; SPD-M20A Detector) using a reverse phase $C_{18}$ column (Mightysil RP-18 GP, 250 × 4.6 mm, Kanto Chemical, Japan) connected to a UV detector (270 nm) and the mass of the products was detected using a high-resolution QTOF-ESIMS [ACQUITY (UPLC, Waters Corp., USA)-SYNAPT G2-S (Waters Corp., USA)]. Purification of compounds was carried out by preparative (prep)-HPLC with a $C_{18}$ column (YMC-Pack ODS-AQ (250 × 20 mm I. D., 10 µm) connected to a UV detector (270 nm) using a 36 min binary program with ACN 20 % (0–5 min), 40 % (5–10 min), 40 % (10–15 min), 90 % (15–25 min), 90 % (25–30 min), and 10 % (30–35 min) at a flow rate of 10 mL/min.

## Fermentation conditions

A glass autoclavable self-controlled fermentor system (Biotron, Korea) was used for large scale analysis and production of the genistin and daidzin as well as their malonylated derivatives. The fermentation was carried out in 3 L of TB medium. Inoculum for the fermentation was prepared in 300 mL volumes in flasks containing TB medium with appropriate antibiotics wherever required. The culture was grown overnight at 37 °C at 220 rpm in a shaking incubator. The inoculum was added in the fermentor containing 3 L of autoclaved medium aseptically. The pH meter and dissolved oxygen (DO) probe were calibrated according to the manufacturer's protocol. The pH was maintained at 7.0 throughout the process by commercially available ammonium hydroxide (28 %). The DO level was maintained above 95 % in the beginning and was never <20 % during the experiment. When the optical density at 600 nm was above 5, the culture was induced by 0.5 mM IPTG and temperature was lowered to 25 °C. Incubation was continued until 48 h. After 5 h of culture induction, 200 µM of the final concentration of genistein (dissolved in 20 % dimethylsulfoxide) was added to the culture. The culture was supplied with 20 mL sterile glucose solution (600 g/L) every hour until 30 h after the start of the feeding of substrate. Samples were taken at 12 h intervals and checked for the quantification of the product formation for 60 h. Then the culture broth was centrifuged and the supernatant was extracted twice with equal volumes of ethyl acetate. The

products were analyzed by HPLC and used for purification. The same procedure was used for production of daidzein derivatives.

### Cell culture and cell proliferation assay

Early passages (passages 4–8) of human umbilical vein endothelial cells (HUVECs) were grown in endothelial growth medium-2 (EGM-2, Lonza, Walkersville, MD, USA) supplemented with 10 % fetal bovine serum (FBS, Invitrogen, Grand Island, NY, USA). B16F10 melanoma cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10 % FBS. AGS gastric cancer cells were grown in RPMI 1640 medium (Invitrogen) containing 10 % FBS. All cells were maintained at 37 °C in a humidified 5 % $CO_2$ incubator. For the cell proliferation assay, cells seeded at $2 \times 10^3$ cells/well in 96-well plates (SPL Lifesciences, Korea) were treated with each compound at various concentrations for 72 h. Cell growth was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.

### Chemoinvasion assay

The invasiveness of endothelial cells was determined in vitro using a Transwell chamber system with polycarbonate filter inserts with a pore size of 8.0 μm. Briefly, the lower side of the filter was coated with 10 μL of gelatin (1 mg/mL), and the upper side was coated with 10 μL of Matrigel (3 mg/mL). HUVECs ($1 \times 10^5$ cells) were placed in the upper chamber of the filter, and each compound was added to the lower chamber in the presence of VEGF (30 ng/mL). The chamber was incubated at 37 °C for 18 h, and then the cells were fixed with methanol and stained with hematoxylin and eosin. The total number of cells that invaded the lower chamber of the filter was counted using an optical microscope (Olympus) at 160× magnification.

### Statistical analysis

The student's *t* test was performed on the biological replicate to determine the statistical significance of the difference between control and experiment samples at each time point. Differences with $P$ value <0.05 were considered statistically significant. Biological activity results are expressed as the mean ± SE.

### Water solubility determination

For water solubility determination, preparative HPLC purified and dried genistin and daidzin were extracted with equal volume (100 μL) of water and ethyl acetate in equimolar concentration (2 mM) and vortexed and centrifuged at 3,000 rpm for 10 min. Aliquots (50 μL) were removed from each water and ethyl acetate layer, and the aliquots were analyzed directly via HPLC as previously described. The HPLC peaks were calculated on the basis of total peak area.

## Results

### Improvement of intracellular UDP-glucose and malonyl-CoA

Since a low concentration of the intracellular UDP-glucose pool is a major barrier for large scale production of glycosylated flavonoids, we undertook the improvement of the intracellular UDP-glucose concentration by using metabolically engineered *E. coli* mutant strain overexpressing galU gene (*E. coli* M2GG). Similarly, the intracellular malonyl-CoA concentration was improved by overexpressing the acyl-CoA carboxylase α and β subunits (*nfa*9890 and *nfa*9940), biotin ligase (*nfa*9950), and acetyl-CoA synthetase (*nfa*3550) from *N. farcinia.* The increase in products of the glyosylated derivatives of genistein and daidzein by 1.25-fold and 1.30-fold respectively and almost twofold increase in malonylated isoflavonoid glucosides indicates that our metabolic work proved to enhance the donors inside the *E. coli.*

### Detection and identification of products

The production of genistin, daidzin and their respective derivatives in crude extracts was analyzed by HPLC–PDA ($t_R$: 10.81, 8.42, 8.98 and 9.42 min for genistein, genistin, genistin 6″-*O*-malonate, and genistin 6″-*O*-acetate, respectively, and $t_R$: 9.95, 7.75, 8.43 and 8.71 min for daidzein, daidzin, daidzin 6″-*O*-malonate, and daidzin 6″-*O*-acetate, respectively) (Fig. 2), and high-resolution HPLC–PDA-QTOF-ESI/MS ($m/z^+$~$[M + H]^+$ 433.1135, 519.1129, 475.1240, 417.1213, 503.1190, and 459.1303 for genistin, genistin 6″-*O*-malonate, genistin 6″-*O*-acetate, and daidzin, daidzin 6″-*O*-malonate, and daidzin 6″-*O*-acetate, respectively) (Fig. S1 and S2).

### Enhancement of genistin and daidzin production in *E. coli*

*E. coli* expressing pET-15b-GmIF7GT converted isoflavonoids into their respective glycosides (genistin and daidzin). For the higher bioconversion of isoflavonoids to glycosylated metabolites, we analyzed the biotransformation kinetics of isoflavonoids with *E. coli* BL21 (DE3) expressing pET-15b-GmIF7GT. The concentration of the substrate and time were optimized by carrying out the

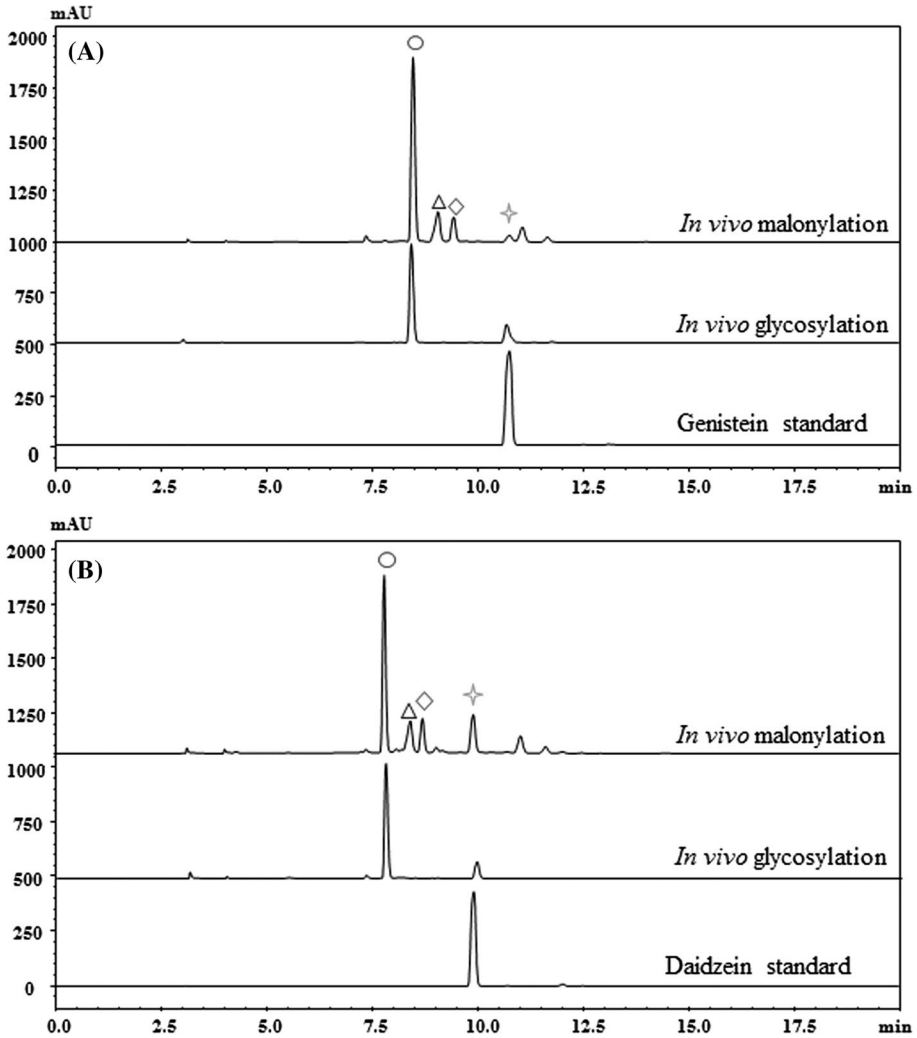



**Fig. 2** HPLC chromatogram of genistein and daidzein reaction product with GmIF7GT and GmIF7MaT. The $t_R$ of genistein and daidzein standard are 10.81 and 9.95 min, respectively, genistin (genistein-7-*O*-β-D-glucoside) and daidzin (daidzein-7-*O*-β-D-glucoside) are 8.42 and 7.75 min, respectively, expected malonylated derivatives of genistin and daidzin are 8.98 and 8.43 min, respectively, and expected acetylated derivatives are 9.4 and 8.7 min, respectively. Substrate, glucoside, malonate, and acetate produced are indicated by *star*, *circle*, *triangle*, and *diamond*, respectively

reactions at different concentrations. 100, 200, 300, 400, 500, and 1,000 μM concentrations of genistein and daidzein were prepared in DMSO and were separately added to the induced culture of the recombinant host. Equal volume of samples was taken and analyzed at 12 h intervals. The time and concentration dependent study showed the highest concentration of the product (140 μM of genistin and 124 μM of daidzin) at 48 h when 200 μM of each was supplemented (Fig. 3). The 200 μM concentration of each substrates and 48 h incubation time were used for the comparative study of the bioconversion in three different media (LB, TB, and M9). The comparative study showed that highest bioconversion was recorded in the TB medium (82 % conversion of genistein and 74.25 % conversion of daidzein. This yield data represent 1.2-fold higher than LB and 1.54-fold higher than the M9 medium for genistein. Similiarly, for daidzein the result represents 1.18-fold higher than LB and 1.56-fold higher than the M9 medium (Fig. 4a, b). The monitoring of optical cell density ($OD_{600nm}$) of the bioconversion reaction in different media revealed the highest cell density in TB medium. The highest bioconversion rate could be attributed to the comparative higher cell growth. Thus optimized concentration of all substrates, incubation time, medium (TB supplemented with 1 % mannitol, 1 % glucose and 1 % glycerol), and mutant strain *E. coli* M2GG were selected for the scale up of the bioconversion reaction in 3 L fermentor.

## Scale up for production using a fermentor

*E. coli* M2GG was cultured in a 3 L fermentor in which the culture conditions were maintained throughout the synthesis of the compound, as described in "Materials and methods". Genistein and daidzein were added to separate 3 L fermentors containing *E. coli* M2GG. The culture medium was harvested at a regular interval of 6 h and analyzed by HPLC to monitor the conversion of genistein and daidzein. Approximately 85 % of the substrates were converted into glycosylated isoflavonoids within 48 h of incubation producing 175.7 μM (75.90 mg/L) of genistin and 162.2 μM (67.65 mg/L) of daidzin (Fig. 4c, d).

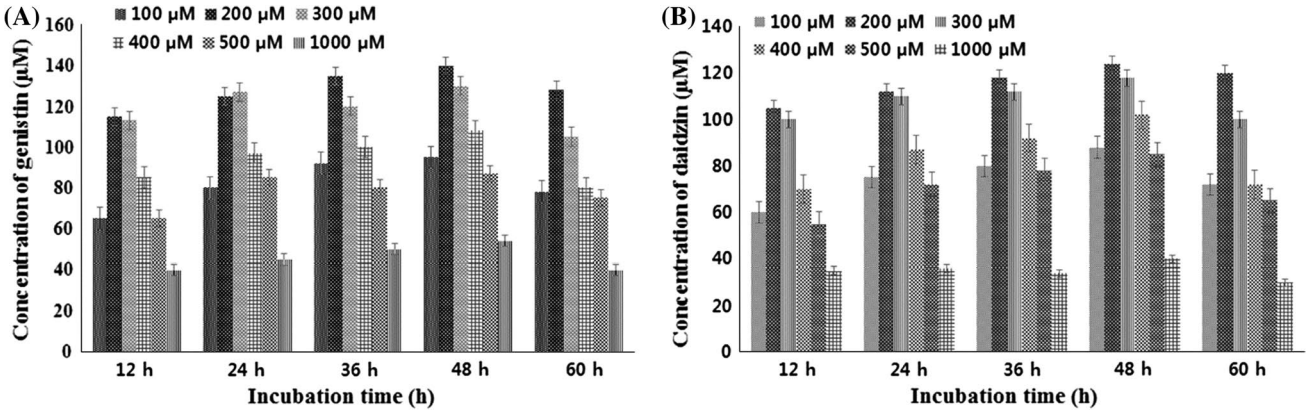



**Fig. 3** Production profile by recombinant host *E. coli* BL21 harboring GmIF7GT upon supplementation of various concentrations of **a** genistein and **b** daidzein in LB medium at different time intervals in a 50 mL culture flask. The experiments were performed in triplicate and repeated three times. *Error bars* indicate standard deviations

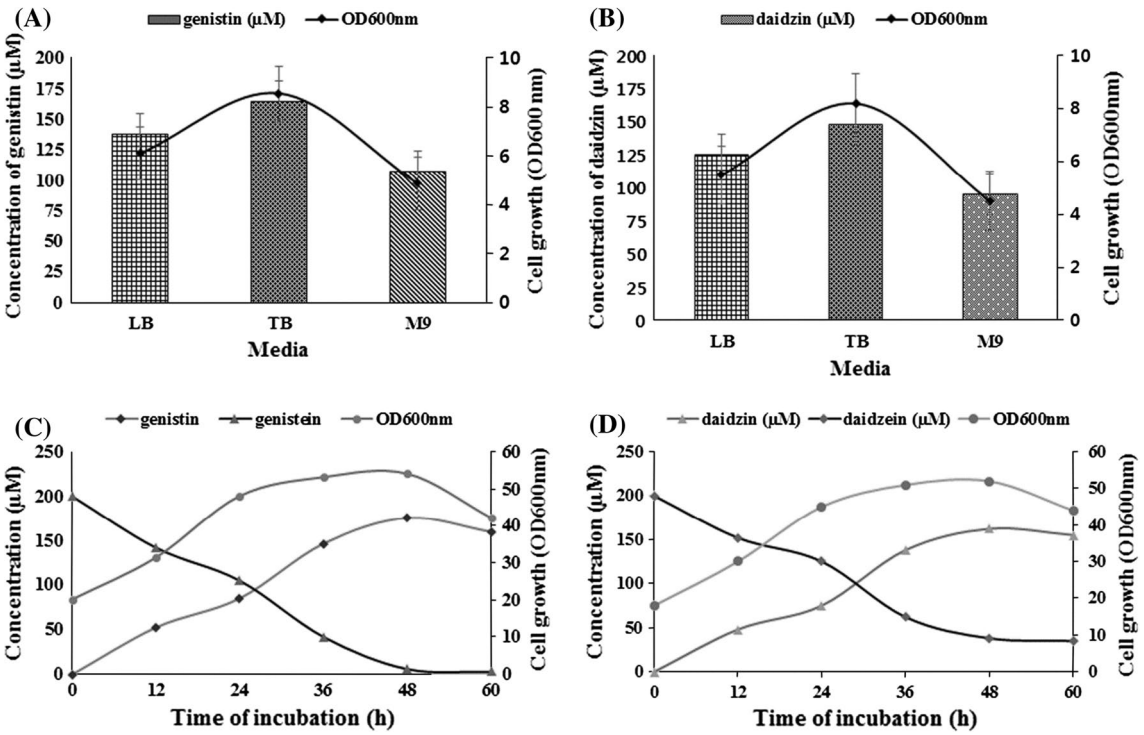



**Fig. 4** Comparison of the production of **a** genistin **b** daidzin in three different commercial media (LB, TB, and M9 minimal salt medium) at 48 h of incubation when 200 µM of genistein and daidzein were respectively supplemented in a 50 mL of culture flask. Large scale bioconversion using fermentor under control conditions of temperature, pH, and dissolved oxygen in TB medium. 200 µM of **c** genistein **d** daidzein were supplied, respectively, for the biotransformation. The experiments were performed in triplicate and repeated three times. *Error bars* indicate standard deviation

## Production of malonylated isoflavonoid glucosides

To carry out both the specific malonylation at the C-6″ position of glucose and the general production of malonylated isoflavonoid glucosides, the GmIF7MaT gene encoding malonyl-CoA: isoflavone 7-*O*-glucoside-6″-*O*-malonyltransferase from *Glycine max* was cloned in the pET-28a expression vector to construct the pET-28a-GmIF7MaT expression recombinant plasmid. Recombinant plasmids pET-15b-GmIF7GT, pET-28a-GmIF7MaT, and pACYC-104 were transformed in host strains to create *E. coli* M1GM and *E. coli* M1GMM as required (Table 1). At 200 µM of each substrate, 14.2 µM

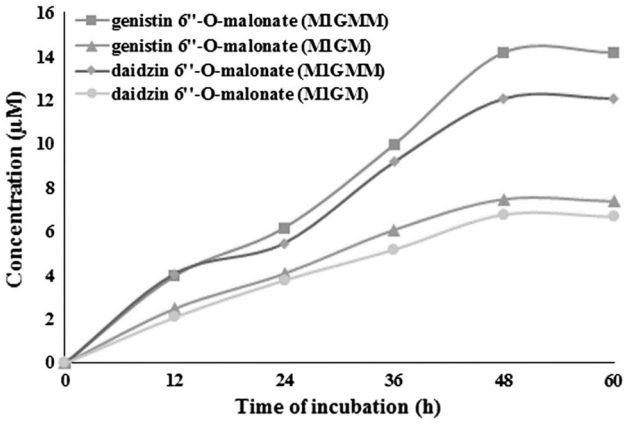



**Fig. 5** Large-scale bioconversion using fermentor at optimized conditions. Time dependent conversion of genistein to genistin 6″-*O*-malonate and daidzein to daidzin 6″-*O*-malonate in fermentor in TB medium supplemented with 1 % glucose 1 % mannitol and 1 % glycerine. 200 μM of each substrate was supplied for the reaction. The experiments were performed in triplicate and repeated three times

(7.2 mg/L) of genistin 6″-*O*-malonate and 12.1 μM (6.03 mg/L) of daidzin 6″-*O*-malonate were produced in TB media (Fig. 5) when the *E. coli* M1GMM strain was used, which was almost twofold higher than the metabolites produced by *E. coli* M1GM strain.

### Biological activity of isoflavonoids and derivatives

Previous in vitro and in vivo studies have shown that the anti-cancer effects of isoflavonoids are associated with their suppressive activities on cancer cell proliferation and angiogenesis. We thus evaluated the effects of genistein, daidzein, their glycosylated forms (genistin and daidzin), and also methylated forms (biochanin A and formononetin) on the proliferation of B16F10 melanoma and AGS gastric cancer cells. The cancer cells were cultured for 3 days in the presence of each compound at various concentrations and then the number of viable cells was measured by MTT assay. Among the six compounds tested, genistein most effectively inhibited the growth of B16F10 and AGS, with $IC_{50}$ values of 7.7 and 39.8 μM, respectively (Fig. 6a, b). However, unlike derivatives of genistein (genistin and biochanin A), the glycosylated and methylated forms of daidzein (daidzin and formononetin) exhibited more effective growth inhibition than aglycon, daidzein (Table 2).

We next investigated the effects of the isoflavonoids and their derivatives on key angiogenic processes by endothelial cells, such as cell proliferation and invasion. As shown in Fig. 6c, six compounds dose-dependently inhibited the proliferation of human umbilical vein endothelial cells (HUVECs). Although the glycosylated form of genistein (genistin) exerted less influence than genistein, that of daidzein showed more potent inhibition activities for the growth of HUVECs. Notably, methylated form of daidzein, formononetin, also exhibited a significant anti-proliferative activity with an $IC_{50}$ of 15.4 μM, suggesting that formononetin may possess the prominent anti-angiogenic activity by specific inhibition of endothelial cell growth.

The anti-angiogenic activity of the glucoside conjugates of genistein and daidzein was further assessed using in vitro angiogenesis assay. Since endothelial cell invasion is a crucial step for the spreading and migration of cells, the inhibition of this step has been considered as an important property for anti-angiogenic agents. Vascular endothelial growth factor (VEGF) was used as a chemoattractant or an angiogenic factor. Serum-starved HUVECs were stimulated by VEGF with or without genistin or daidzin and in vitro chemoinvasion assay was performed. As shown in Fig. 7, genistein and daidzein significantly inhibited VEGF-induced invasion of HUVECs in a dose-dependent manner. Interestingly, like genistein and daidzein, their glucosides genistin and daidzin effectively suppressed the invasion of HUVECs induced by VEGF at the same concentrations.

### Water solubility determination

For the water solubility determination, preparative HPLC purified and dried genistin and daidzin as well as aglycones were extracted with equal volume of water and ethyl acetate in equimolar concentration, vortexed, and then centrifuged to separate it into two layers. Each water and solvent layer was directly analyzed using HPLC, as described previously. The water solubility of glycosylated compounds has approximately fivefold greater water solubility in comparison to substrates. When equal concentrations of genistein and genistin was mixed together in equal volume of water and ethyl acetate, almost no substrate was extracted in the water fraction as shown in the HPLC chromatogram (Fig. S3). Similar results were obtained from daidzein and its glycosylated derivative. Thus, produced glycosylated derivatives of isoflavonoids (genistin and daidzin) have higher auqeous solubility than their parent compound.

## Discussion

Isoflavonoids such as genistein and daidzein are widely used in the pharmaceutical industries. The efficacy of their functions can be enhanced by modifications such as glycosylation and malonylation. The primary source for isoflavonoids and their glycosylated and malonylated derivatives still depends heavily on plant sources. Efficient production of glycosylated and malonylated derivatives of isoflavonoids from plants is challenging, tedious, costly,

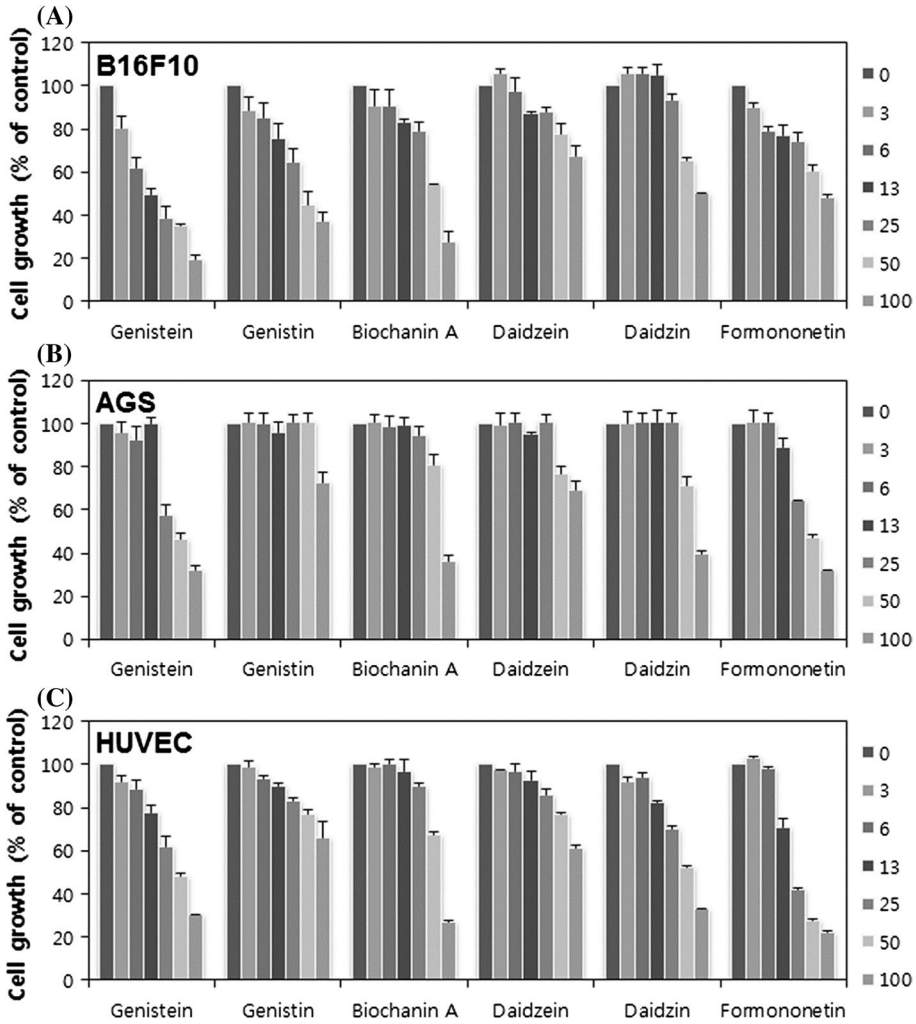



**Fig. 6** Effects of genistin and daidzin on cell proliferation. Cancer (**a**, **b**) and endothelial (**c**) cells were treated with various concentrations (0–100 μM) of each compound for 72 h and cell growth was measured using MTT colorimetric assay. Each value represents mean ± SE from three independent experiments

**Table 2** $IC_{50}$ (μM) value of genistein, genistin, biochanin A, daidzein, daidzin and formononetin on cell proliferation. Each value represents mean ± SE from three independent experiments

| $IC_{50}$ (μM) | B16F10 | AGS | HUVEC |
|---|---|---|---|
| Genistein | 7.7 | 39.8 | 48.3 |
| Genistin | 28.4 | >100 | >100 |
| Biochanin A | 52.9 | 84.4 | 72.5 |
| Daidzein | >100 | >100 | >100 |
| Daidzin | 81.0 | 75.7 | 48.2 |
| Formononetin | 79.9 | 42.3 | 15.4 |

labor-intensive purification, and difficult to scale up. To overcome these problems, metabolic engineering and synthetic biology have been applied to enhance the efficient production of flavonoid glycosides and other derivatives [15, 22]. *E. coli* has well-characterized physiology, a highly tractable genetic system, and can be metabolically manipulated as required. These properties make *E. coli* one of the most widely used microorganism for the production of natural products and their derivatives [2, 6, 13, 16].

One major aspect of our research is the enhancement of the UDP-glucose pool inside the host strain. To achieve that, *E. coli* mutant strain overexpressing *galU* was used. These deletions led to the increased accumulation of glucose-1-phosphate, whereas over-expression of genes increased the endogenous UDP-glucose inside *E. coli.* This was clearly indicated by the 1.25- and 1.30-fold increase in the glyosylated derivatives of genistein and daidzein, respectively, as compared to the wild type strain when GmIF7GT was used as a glycosyltransferase.

Another important aspect of this research is the increase of malonyl-CoA pool inside the host strain. *E. coli* generates very low levels of intracellular malonyl-CoA [33] which limit the wide utilization of this host for commercial-scale production of malonylated derivatives of flavonoids and other important polyketides. Furthermore ACCase, which is crucial for the anabolism of malonyl-CoA and fatty acids, was also suggested to be the major

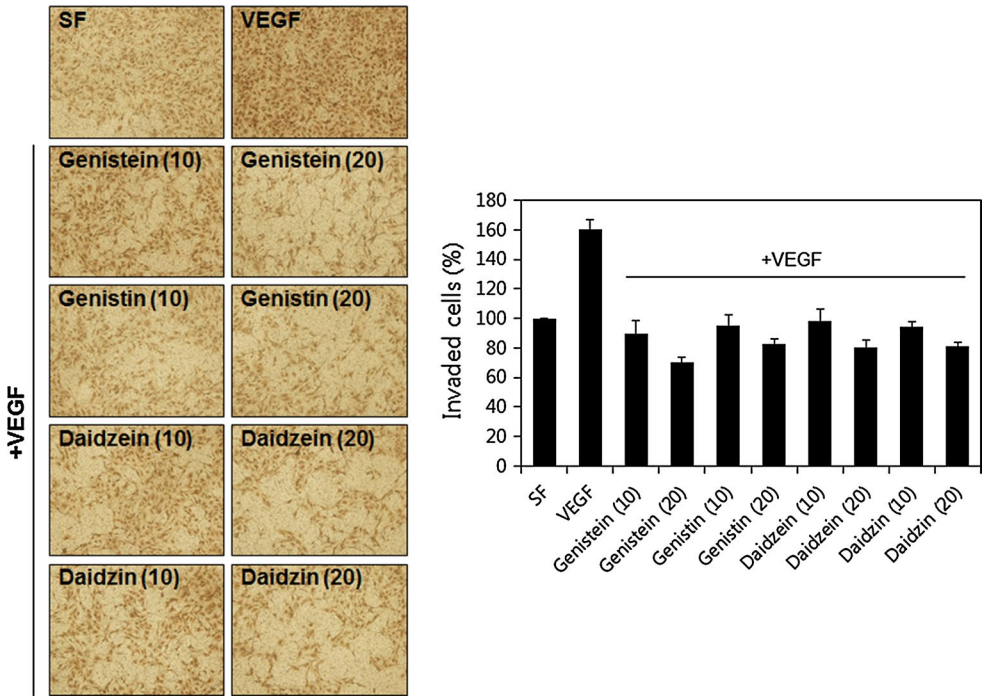



**Fig. 7** Effects of genistin and daidzin on the invasion of HUVECs. Serum-starved HUVECs were stimulated with VEGF (30 ng/mL) in the presence or absence of each compound (10 and 20 μM). The basal level of invasiveness of HUVECs that were incubated in serum-free (SF) media was normalized to 100 %. Each value represents mean ± SE from three independent experiments

rate-controlling step for production of high-valued compounds derived from malonyl-CoA [24]. In the present study, the intracellular malonyl-CoA pathway was engineered by overexpressing ACC and ACS genes from *N. farcinia.* In the recombinant strain *E. coli* pACYC104, Malla and his group found that the concentration of malonyl-CoA increased about 2.3-fold, i.e., from 0.88 to 2.01 nmol/mg DCW at 6 h of the growth phase [17]. The addition of 1 % glucose, 1 % mannitol and 1 % glycerol in the culture media proved to be fruitful in producing more glycosylated products, so we applied the same formulation in the fermentor scale [23]. The affectivity of glucose in increasing the intracellular acetyl-CoA and malonyl-CoA concentration via the glycolysis pathway has already been reported by Takamura and his group [33]. Importantly, the threshold amount of malonyl-CoA in *E. coli* is attributed to the complexity of fatty acid metabolism. In this case, the fatty acid synthases mediated the conversion of malonyl-CoA into acetyl-CoA. To circumvent the depletion of malonyl-CoA, Leonard and his group reported that the inhibition of fatty acid biosynthesis by cerulenin increased the production levels of flavonoids to over 900 % [13]. Strong metabolic channeling of carbon flux towards fatty acid biosynthesis and systematically repressing FabB and FabF with a specific inhibitor, is the major competitive step in enhanced production of malonylated isoflavonoid glucosides.

In the present study, we also found that genistin and daidzin may have powerful anti-cancer activity by inhibiting the invasive potential of endothelial cells, which is crucial for tumor growth and metastasis. Our data provide that the biotransformation of isoflavones by glycosylation not only contributes to the improvement of water solubility and stability but also improves the pharmacological properties for cancer treatment. These results indicate that genistin and daidzin have biological activity which do not require initial hydrolysis to genistein and daidzein, respectively. Furthermore isoflavones biochanin-A and formononetin also showed better anti-angiogenic activities in vitro suggesting that methylation of isoflavones could cause better pharmacological activity. The structural differences of the isoflavonoids may be responsible, in part, for these results. Further research will be carried out in future in this area by our group as methylation is significant in improving the stability of flavonoids [11]. Findings from the Choi group have found that although genistin appeared to be less potent than genistein, genistin might induce cell arrest not only at the G1-phase, but also at the G2/M phase via multiple pathways. Combined therapy of genistein and genistin [3] or daidzein and daidzin or genistin and daidzin [37] may be more effective than aglycone alone.

## Conclusion

In conclusion, we have successfully generated pharmaceutically important derivatives of genistein and daidzein in our lab on the fermentor scale. These results indicated the positive effects of gene deletion and overexpression of genes involved in the biosynthetic pathway of required precursors. Though the isoflavonoid glycosides were

quantitatively dominating over the malonates, overexpression of genes involved in the production of malonyl-CoA precursors slightly enhanced the production of malonylated cum glucosylated isoflavonoids. Unfortunately, genistin 6″-*O*-acetate and daidzin 6″-*O*-acetate might have been formed from their respective malonates by the loss of carbon dioxide and the dominating accumulation of glucosides inside the cell may be the reversible decomposition of genistin 6″-*O*-malonate and daidzin 6″-*O*-malonate back to genistin and daidzin respectively, suggesting that the malonate degradation pathway is more pronounced under these growth conditions.

The ease of working with an *E. coli*, requiring only inexpensive carbon sources, makes the engineered strain practically useful and flexible whole-cell catalyst for obtaining industrial scale of valuable isoflavonoid derivatives. Advances in metabolic pathway and strain engineering may pave the way for sustainable production of isoflavonoid derivatives possessing anti-cancer activity with improved solubility and stability for drugs and cosmetics industry.

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