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Biosynthesis of resveratrol derivatives and evaluation of their anti-inflammatory activity

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Abstract

Resveratrol is a typical plant phenolic compound whose derivatives are synthesized through hydroxylation, *O*-methylation, prenylation, and oligomerization. Resveratrol and its derivatives exhibit anti-neurodegenerative, anti-rheumatoid, and anti-inflammatory effects. Owing to the diverse biological activities of these compounds and their importance in human health, this study attempted to synthesize five resveratrol derivatives (isorhapontigenin, pterostilbene, 4-methoxyresveratrol, piceatannol, and rhapontigenin) using *Escherichia coli*. Two-culture system was used to improve the final yield of resveratrol derivatives. Resveratrol was synthesized in the first *E. coli* cell that harbored genes for resveratrol biosynthesis including *TAL* (tyrosine ammonia lyase), *4CL* (4-coumaroyl CoA ligase), *STS* (stilbene synthase) and genes for tyrosine biosynthesis such as *aroG* (deoxyphosphoheptonate aldolase) and *tyrA* (prephenate dehydrogenase). Thereafter, culture filtrate from the first cell was used for the modification reaction carried out using the second *E. coli* harboring hydroxylase and/or *O*-methyltransferase. Approximately, 89.8 mg/L of resveratrol was synthesized and using the same, five derivatives were prepared with a conversion rate of 88.2% to 22.9%. Using these synthesized resveratrol derivatives, we evaluated their anti-inflammatory activity. 4-Methoxyresveratrol, pterostilbene and isorhapontigenin showed the anti-inflammatory effects without any toxicity. In addition, pterostilbene exhibited the enhanced anti-inflammatory effects for macrophages compared to resveratrol.

Keywords: Anti-inflammatory activity, Hydroxylation, *O*-methylation, Resveratrol derivatives

Introduction

Resveratrol is a natural phenolic compound that belongs to the stilbenoid group. Resveratrol and its glucoside (resveratrol 3-*O*-glucoside, piceid) are the two major stilbenoids found in nature [1]. Grape is one of the primary sources of resveratrol and its derivatives [2]. Major resveratrol derivatives are synthesized via hydroxylation, glycosylation, *O*-methylation, and prenylation [3]; resveratrol derivatives from oligomerization have been reported in plants [4]. Resveratrol is synthesized from tyrosine through deamination by tyrosine ammonia lyase (TAL), Co-A attachment by 4-hydroxycinnamoyl-CoA ligase (4CL), and condensation with three molecules of malonyl-CoA by stilbene synthase (STS) [5]. Genes that encode each step of resveratrol biosynthesis from tyrosine have been cloned and characterized [3]. Among them, gene encoding STS, a type III polyketide synthase [6], has been cloned in various plants, including *Arachis hypogaea* (groundnut; [7]), *Pinus densiflora* [8], and *Vitis vinifera* [6]. Although plants are known to be a good source, resveratrol has been synthesized in a microbial system by introducing genes associated with biosynthesis (reviewed by [3]). Previous studies included selection of the best combination of *TAL*, *STS*, and *4CL*,

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and supplementation with additional *p*-coumaric acid and malonyl-CoA [3]. These approaches included pathway engineering to enhance the supplementation of tyrosine and malonyl-CoA or modulation of the entire pathway. Using *Saccharomyces cerevisiae*, 415.7 mg/L of resveratrol was synthesized from glucose via tyrosine and malonyl-CoA overproduction in fed-batch fermentation [9]. Implementing the pull–push block strategy, final yield of resveratrol in *S. cerevisiae* increased up to 800 mg/L [10]. In *Escherichia coli*, the resveratrol synthesis pathway was introduced, following different strategies, to increase the pool of malonyl-CoA; the resulting strains produced 304.5 mg/L resveratrol from glucose [11] and 238.71 mg/L resveratrol from tyrosine [12]. Not only resveratrol but also its derivatives could be synthesized, given the regioselective modification enzymes are available. Based on the structure of resveratrol, biological modifications are possible through *O*-methylation and hydroxylation. The three possible modification products include mono-*O*-methylation, di-*O*-methylation, or three-*O*-methylation. To date, *E. coli* has been engineered to produce 34.0 mg/L of pinostilbene (3-methylated resveratrol) and 0.16 mg/L of pterostilbene (3′,5′-dimethylated resveratrol) from 1 mM resveratrol [13]. Mixture of mono-, di-, and tri-*O*-methylated resveratrol was synthesized from glucose in *E. coli* [14]. On supplementing the culture medium with methionine to enhance the supply of *S*-adenosyl-*L*-methionine, 33.6 mg/L of pterostilbene was synthesized in *E. coli* [15]. Biosynthesis of pinostilbene (5.5 mg/L) and pterostilbene (34.9 mg/L) was also achieved in *S. cerevisiae* [10]. Piceatannol was synthesized from glucose in *E. coli* with a yield of 21.5 mg/L [16]. Overexpression of acetate and malonate assimilation pathways along with additional supply of malonate in the culture medium yielded 124 mg/L of piceatannol (3-hydroxyresveratrol) in *E. coli* [17].

Extensive research on the derivatives of resveratrol stems from their biological significance. Studies demonstrated that resveratrol downregulated the inflammatory response [18, 19] leading to decreased inflammation [20, 21]. This anti-inflammatory effect of resveratrol was considered one of the potential therapeutic strategies for treating various chronic diseases, such as cancer, diabetes, and neurodegenerative and cardiovascular disorders (reviewed by [19]).

Resveratrol derivatives that were modified by *O*-methylation and/or hydroxylation showed enhanced biological effects compared to resveratrol. Pterostilbene was reported to exhibit anticancer, anti-inflammatory, and antioxidant effects [22–24]. It also increasingly inhibited induction of inflammatory genes, such as inducible nitric oxide synthase and cyclooxygenase 2 [23]. Apart from the antioxidative and antiproliferative activities [25], piceatannol also demonstrated anticancer effect [26]. In addition, this compound was studied for its anti-inflammatory effects as well [27–29]. Rhapontigenin demonstrated anticancer, anti-inflammatory, cardioprotective, antiallergic, and antithrombotic effects [24, 30–32], while isorhapontigenin (3-methoxyresveratrol) showed anticancer [33], antioxidative [34], and anti-inflammatory activities. Compared to resveratrol, oral bioavailability of isorhapontigenin was much higher [35].

Resveratrol derivatives are highly valued owing to their diverse biological activities. Hence, in this study, attempt was made to synthesize resveratrol and five resveratrol derivatives using *E. coli* (Fig. 1). Simple modification reactions found in nature were mimicked by introducing the corresponding genes into a heterologous system. In this regard, the two-culture system was found to be effective for the modification reactions; one for resveratrol biosynthesis and the other for modification of resveratrol to obtain resveratrol derivatives. In addition, anti-inflammatory activities of the synthesized resveratrol derivatives were also evaluated.

Materials and methods

Materials

Lipopolysaccharides (LPS) from *Pseudomonas aeruginosa* 10 was purchased from Sigma Aldrich (St. Louis, MO, USA). The mouse tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) enzyme-linked immunosorbent assay (ELISA) kits were purchased from BD Biosciences (Franklin Lake, NJ, USA). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), penicillin/streptomycin (P/S), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). A micro-bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fisher Scientific (Rockford, IL, USA).

Constructs

STS (DQ406692) was cloned from *Vitis vinifera* using reverse transcription polymerase chain reaction (RT-PCR). The primers used were ATGAATTCGATGGC TTCAGTCGAGGAATTT (EcoRI site is underlined) and ATGTCGACTTAATTTGTAACCATAGGAATG (SalI site is underlined). The PCR product was sequenced and subcloned into the EcoRI/SalI sites of pCDF-Duet (pC-VvSTS). R4CL [36] containing the ribosomal binding site and SalI site at the 5′ end and NotI site was subcloned into SalI/NotI site of pC-VvSTS. The resulting construct was named pC-VvSTS-Os4CL. SeTAL was cloned previously in NdeI/KpnI site of pCDF-Duet1 [37]. Using pC-VvSTS-Os4CL as a template, PCR was carried out with forward primer of VvSTS and reverse primer of Os4CL containing HindIII. The resulting PCR product was

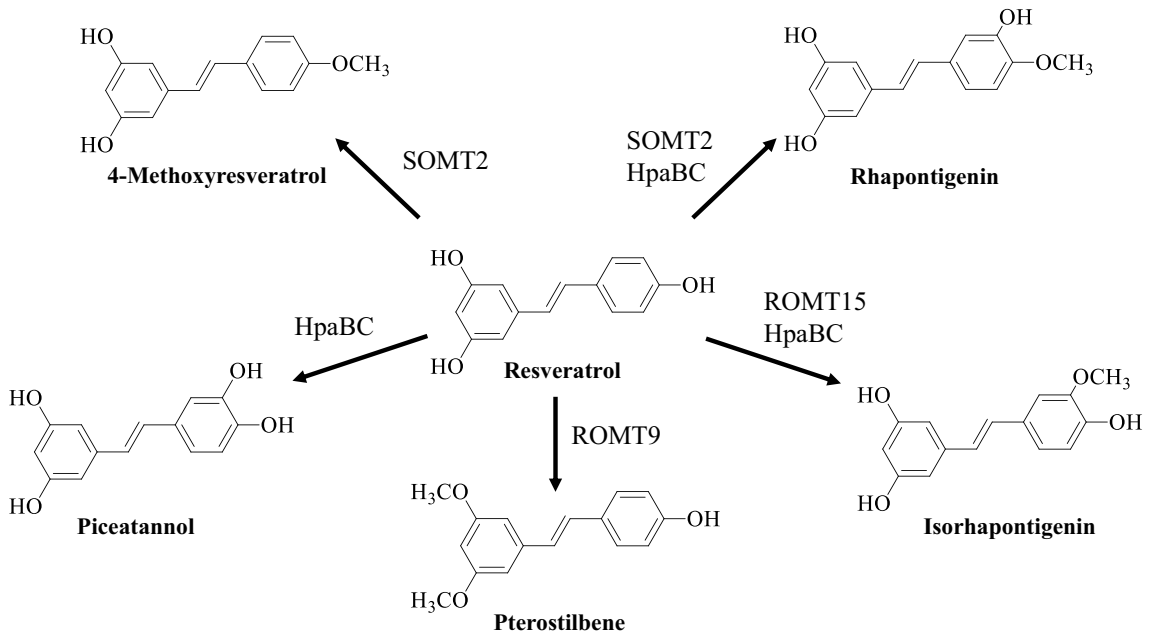

Fig. 1 Structures of the resveratrol derivatives synthesized in this study. *SOMT2*, *ROMT9*, and *ROMT15* encode *O*-methyltransferases. *HpaBC* encodes a hydroxylase

digested with EcoRI/HindIII and subcloned into EcoRI/HindIII of pC-SeTAL (pC-VvSTS-Os4CL-SeTAL).

ROMT15 and *HpaBC* were cloned previously [38, 39]. *ROMT15* was subcloned into EcoRI/NotI sites of *ROMT15* (pE-ROMT15) and *HpaBC* was subcloned into NdeI/XhoI site of pE-ROMT15 (pE-ROMT15-HpaBC). pG-ROMT9 and pG-SOMT2 were made previously [40, 41]. pE-SOMT2-HpaBC was constructed in a way similar to that of pE-ROMT15-HpaBC. Other genes used in this study were cloned previously [42–44] and reconstructed for use in the current study. Briefly, an internal BamHI site of $tyrA^f$ was eliminated by site-directed mutagenesis, without changing the amino acid sequence. It was then subcloned into NdeI/KpnI site of pACYC-duet-1 (pA-tyrA^f). Other genes (*aroG*, $aroG^f$, $aroG^f$-*ppsA-tktA*, *aroL-*$aroG^f$*-ppsA-tktA*, *aroC-aroA-aroL-aroE-aroD-aroB-*$aroG^f$*-ppsA-tktA*, or *aroC-aroA-aroL-ydiB-aroD-aroB-*$aroG^f$*-ppsA-tktA*) were subcloned into the BamHI/NotI sites of pA-tyrA^f. The constructs and *E. coli* strains used in this study were listed in Table 1.

Synthesis of resveratrol derivatives

E. coli BL21 (DE3) was used as the host for synthesis of resveratrol derivatives. *E. coli* transformant was grown in Luria–Bertani (LB) broth with proper antibiotic(s) at 37 °C for 18 h. The culture was then inoculated into a fresh medium and allowed to grow at 37 °C until an optical density value of 1 at 600 nm was achieved. Cells were again resuspended in M9 medium containing 1% yeast extract and incubated at 30 °C for 24 h. The culture was extracted with three volumes of ethyl acetate. After being harvested, dried, and dissolved in dimethyl sulfoxide, the organic layer was analyzed using high performance liquid chromatography (HPLC). For the modification reaction of resveratrol, culture from the B-RS2 strain, initially used for resveratrol production (See Results and Discussion), was centrifuged. The supernatant was then harvested and boiled to destroy the antibiotics. Thereafter, it was mixed with the *E. coli* transformant harboring the construct for synthesis of resveratrol derivative. The reaction product was analyzed using HPLC, as described earlier [45]. All experiments were repeated thrice. Resveratrol was used to quantify the synthesized derivatives.

In order to determine the structures of the synthesized resveratrol derivatives, they were purified using thin layer chromatography (TLC; TLC silica gel 60 F254; Millipore, Burlington, MA, USA). The developing solvents used for each compound were as follows: isorhapontigenin (benzene: methanol: acetic acid = 6: 1.5: 0.1), pterostilbene (ethyl acetate: hexane: acetic acid = 1: 1: 0.1), 4-methoxyresveratrol (benzene: methanol = 5: 1), piceatannol (benzene: methanol: acetic acid = 15: 2.5: 0.1), and rhapontigenin (benzene: methanol: acetic acid = 8.5: 1: 0.1). Proton nuclear magnetic resonance spectroscopy (NMR) was carried out as described previously [46]. ^{1}H NMR of isorhapontigenin in methanol-d6: δ 3.90 (3H, s),

Table 1 Plasmids and *Escherichia coli* strains used in this study

Plasmids and *E. coli* strains	Relevant properties and genetic markers	Sources and references
Plasmids		
pETDuet	F1 ori, Amp^r	Novagen
pCDFDuet	CDF ori, Sm^r	Novagen
pACYCDuet	P15A ori, Cm^r	Novagen
pGEX 5X-1	pBR322 ori, Amp^r	GE Healthcare
pC-SeTAL-VvSTS-Os4CL	pCDFDuet carrying *TAL* from *Saccharothrix espanaensis, STS* from *Vitis vinifera* and *4CL* from *Oryza sativa*	This study
pG-ROMT9	pGEX 5X-1 carrying *ROMT9* from *Oryza sativa*	[40]
pG-SOMT2	pGEX 5X-1 carrying *SOMT2* from *Glycine max*	[41]
pG-HpaBC	pGEX 5X-1 carrying *HpaBC* from *E. coli*	[39]
pE-SOMT2-HpaBC	pGEX 5X-1 carrying *SOMT2* from *Glycine max* and *HpaBC* from *E. coli*	This study
pE-ROMT15-HpaBC	pETDuet carrying *ROMT15* from *Oryza sativa* and *HpaBC* from *E. coli*	This study
pA-aroG-tyrA	pACYCDuet carrying *aroG* and *tyrA* from *E. coli*	This study
pA-$aroG^f$-$tyrA^f$	pACYCDuet carrying $aroG^f$ and $tyrA^f$ from *E. coli*	This study
pA-$aroG^f$-$tyrA^f$-ppsA-tktA	pACYCDuet carrying $aroG^f$, $tyrA^f$, *ppsA* and *tktA* from *E. coli*	This study
pA-aroL-$aroG^f$-$tyrA^f$-ppsA-tktA	pACYCDuet carrying *aroL*, $aroG^f$, $tyrA^f$, *ppsA* and *tktA* from *E. coli*	This study
pA-aroC-aroA-aroL-ydiB-aroD-aroB-$aroG^f$-$tyrA^f$-ppsA-tktA	pACYCDuet carrying *aroC, aroA, aroL, ydiB, aroD, aroB,* $aroG^f$, $tyrA^f$, *ppsA* and *tktA* from *E. coli*	This study
pA-aroC-aroA-aroL-aroE-aroD-aroB-$aroG^f$-$tyrA^f$-ppsA-tktA	pACYCDuet carrying *aroC, aroA, aroL, aroE, aroD, aroB,* $aroG^f$, $tyrA^f$, *ppsA* and *tktA* from *E. coli*	This study
Strains		
BL21 (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm lon* (DE3)	Novagen
B-RS1	BL21 harboring pC-SeTAL-VvSTS-Os4CL and pACYCDuet	This study
B-RS2	BL21 harboring pC-SeTAL-VvSTS-Os4CL and pA-aroG-tyrA	This study
B-RS3	BL21 harboring pC-SeTAL-VvSTS-Os4CL and pA-$aroG^f$-$tyrA^f$	This study
B-RS4	BL21 harboring pC-SeTAL-VvSTS-Os4CL and pA-$aroG^f$-$tyrA^f$-ppsA-tktA	This study
B-RS5	BL21 harboring pC-SeTAL-VvSTS-Os4CL and pA-aroL-$aroG^f$-$tyrA^f$-ppsA-tktA	This study
B-RS6	BL21 harboring pC-SeTAL-VvSTS-Os4CL and pA-aroC-aroA-aroL-ydiB-aroD-aroB-$aroG^f$-$tyrA^f$-ppsA-tktA	This study
B-RS7	BL21 harboring pC-SeTAL-VvSTS-Os4CL and pA-aroC-aroA-aroL-aroE-aroD-aroB-$aroG^f$-$tyrA^f$-ppsA-tktA	This study

6.16 (1H, dd, J = 2.1, 2.1 Hz), 6.46 (2H, d, J = 2.1 Hz), 6.77 (1H, d, J = 8.2 Hz), 6.83 (1H, d, J = 16.2 Hz), 6.95 (1H, dd, J = 8.2, 1.8 Hz), 6.96 (1H, d, J = 16.2 Hz), and 7.11 (1H, d, J = 1.8 Hz); ^{1}H NMR of pterostilbene in chloroform-d1: δ 3.83 (6H, 3′/5′-methoxy, s), 6.38 (1H, H-4′, dd, J = 2.2, 2.2 Hz), 6.65 (2H, H-2′/H-6′, d, J = 2.2 Hz), 6.63 (2H, H-3/H-5, d, J = 9.3 Hz), 6.89 (1H, H-β, d, J = 16.3 Hz), 7.02 (1H, H-α, d, J = 16.3 Hz), and 7.39 (2H, H-2/H-6, d, J = 9.3 Hz); ^{1}H NMR of 4-methoxyresveratrol in methanol-d4 (in ppm): δ 3.80 (3H, s), 6.16 (1H, dd, J = 2.1, 2.1 Hz), 6.45 (2H, d, J = 2.1 Hz), 6.84 (1H, d, J = 16.3 Hz), 6.89 (2H, d, J = 8.7 Hz), 6.98 (1H, d, J = 16.3 Hz), and 7.44 (2H, d, J = 8.9 Hz); ^{1}H NMR of piceatannol in deuterated methanol: δ (ppm) 6.97 (d, J = 1.9 Hz, 1H), 6.88 (d, J = 16.2 Hz, 1H), 6.83 (dd, J = 2.0, 8.2 Hz, 1H), 6.74 (d, J = 15.2 Hz, 1H), 6.73 (d, J = 8.2 Hz, 1H), 6.42 (d, J = 2.1 Hz, 2H), and 6.15 (d, J = 2.1 Hz, 1H); ^{1}H NMR of rhapontigenin in methanol-d4 (in ppm): δ 3.86 (3H, s, 4-OCH_3), 6.17 (1H, dd, J = 2.1, 2.1 Hz, H4′), 6.45 (2H, d, J = 2.1 Hz, H2′/6′), 6.79 (1H, d, J = 16.1 Hz, Hβ), 6.89 (1H, d, J = 8.4 Hz, H5), 6.91 (1H, d, J = 16.1 Hz, Hα), 6.94 (1H, dd, J = 8.4, 2.0 Hz, H6), and 7.01 (1H, d, J = 2.0 Hz, H2).

Anti-inflammatory activity

RAW264.7 cells (murine macrophage cell line) were maintained in complete DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37 °C in a humidified atmosphere with 5% carbon dioxide. The cells were plated in 6-well plates at a density of 6×10^5 cells per well and incubated for 24 h prior to treatment. According to a previous study, the cells were pre-treated for 6 h with resveratrol and its derivatives at a concentration of 10 µM. Cells cultured in a complete medium were then treated with LPS (1 µg/mL) for 18 h in the presence of resveratrol derivatives

at the same concentration [47]. Cells treated with LPS (1 µg/mL) alone were used as control. Cell viability was determined based on the amount of protein in the cells [48]. Briefly, after incubation, the cells were washed with DPBS and lysed using a lysis buffer. The cell lysate was centrifuged at 13,000 rpm for 5 min. The total amount of protein in the supernatant was quantified using a BCA protein assay kit, according to the manufacturer's protocol. In order to determine the released cytokine level, supernatants were centrifuged at 13,000 rpm for 10 min at 4 °C to remove the remaining cell debris. Amounts of TNF-α and IL-6 in the supernatants were quantified using an ELISA assay kit, according to the manufacturer's protocol. The data and error bars represent the mean values of independent measurements and the standard deviations for each experiment. Statistical analysis was performed using Student's *t*-test. A p-value < 0.05 was considered statistically significant (95% confidence level).

Results and discussion

Optimization of the construct for resveratrol biosynthesis

Three genes, namely *TAL*, *4CL*, and *STS*, synthesize resveratrol from tyrosine. *E. coli* was transformed with a construct containing all the three genes and the resulting transformant was tested for resveratrol synthesis. The synthesized compound showed a retention time similar to that of resveratrol, indicating successful synthesis of resveratrol. Approximately, 32.0 mg/L of resveratrol was synthesized after 24 h of reaction time. Absence of *p*-coumaric acid indicated that there could be a possibility for increased resveratrol synthesis, provided additional *p*-coumaric acid was added to the medium. Recent report also showed that these three genes were critical for the synthesis of resveratrol [49].

Since *p*-coumaric acid is synthesized from tyrosine, the amount of endogenous tyrosine plays a critical role in determining the final yield of resveratrol. Previously, it has been shown that overexpression of the genes in the shikimate pathway increases the yield by utilizing the intermediates in the shikimate pathway [43, 44, 50]. Herein, seven constructs with different combination of genes in shikimate pathway were tested. Each of the constructs was transformed along with pC-VvSTS-Os4CL-SeTAL into *E. coli* BL21 (DE3) cell. The resulting transformant (B-RS1 ~ 7) was then monitored for resveratrol synthesis. Results revealed that the culture filtrate of each strain not only contains resveratrol but also has *p*-coumaric acid. All the *E. coli* strains, except for the one harboring only pC-VvSTS-Os4CL-SeTAL, exhibited enhanced resveratrol synthesis. Among them, the B-RS3 strain reported highest resveratrol (89.8 mg/L) production and contained two feedback inhibition free genes (*aroG*^{f} and *tyrA*^{f}). The B-RS5 strain synthesized approximately 81.9 mg/L of resveratrol (Fig. 2). The final yield in B-RS3 was higher than that of the study using mutant strain [49].

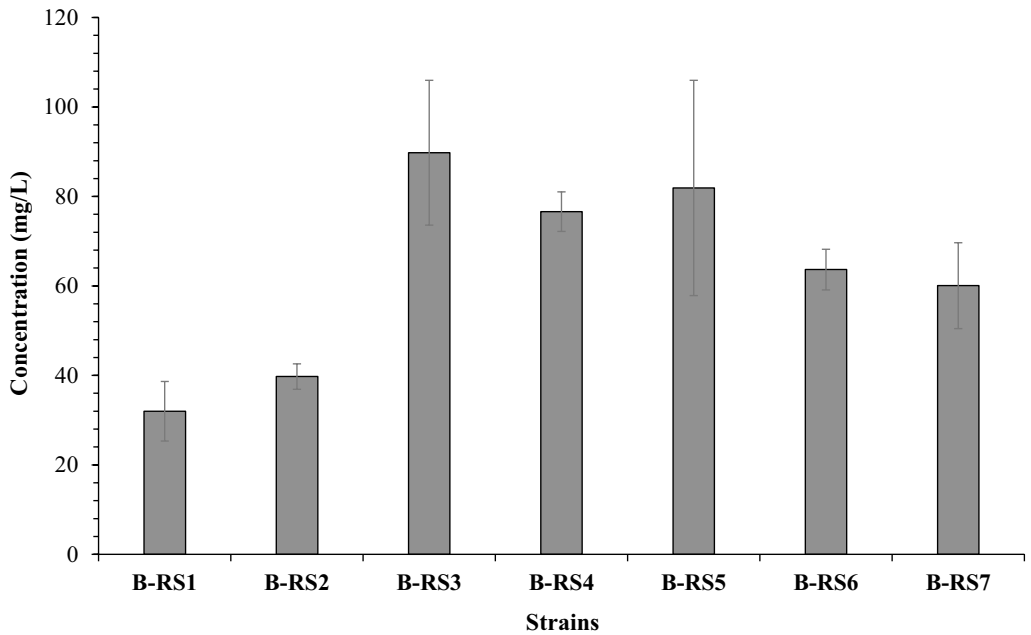

Fig. 2 Comparison of different module constructs for the synthesis of resveratrol. Each strain had genes encoding tyrosine ammonia lyase, 4-hydroxycinnamoyl-CoA ligase, and stilbene synthase. The different shikimate pathway gene modules indicated in Table 1 were introduced

In order to synthesize 4-hydroxycoumarin, quinoline, acridone, and 4-hyroxybenzoic acid in *E. coli*, we used module constructs that were prepared using various combination of genes involved in the shikimate pathway. Balance between the substrate(s) and product resulted in the highest yield. The level of *p*-coumaric acid, a substrate of resveratrol, was highest in the B-RS4 strain, followed by B-RS5 and B-RS3 strains. These three strains showed the highest resveratrol production. However, the best module construct for resveratrol production was different from that for other compound synthesis. This is likely to stem from the differences in substrate conversion capabilities of the downstream gene. The final yield of the current study was not comparable to the previous studies [11, 12]. Earlier *E. coli* was engineered to increase not only tyrosine (substrate for *p*-coumaric acid), but also malonyl-CoA. The current study tested the viability of the module constructs to increase tyrosine production. However, the final goal of this study was to synthesize resveratrol derivatives. Higher yield for resveratrol did not ensure increased production of resveratrol derivatives. Instead, a balance between the upstream and downstream reaction products was critical to determine the final yield. Although the final yield of resveratrol was lower compared to that of the previous studies, it was high enough to synthesize other resveratrol derivatives (See below).

Synthesis of five resveratrol derivatives using a two-culture system

We attempted to synthesize five resveratrol derivatives (Fig. 1). First, we attempted to synthesize pterostilbene in *E. coli*. One additional gene (*ROMT9*) was introduced into the strain B-RS3. It was observed that *E. coli* harboring *ROMT9* converted resveratrol to pterostilbene (data not shown). However, the B-RS3 strain harboring *ROMT9* synthesized resveratrol instead of pterostilbene. It is likely that the function of *ROMT9* was inhibited by some metabolites. Next, a two-culture system, which has been recently implemented for the synthesis of anthocyanin and 4-hydroxy-1-methyl-2-quinolone, was used for the synthesis of resveratrol derivatives [43, 51]. The whole reaction was divided into two steps. In the first step, cells synthesized resveratrol from glucose, while in the second step, they synthesized pterostilbene from resveratrol. The B-RS3 strain was used to synthesize resveratrol (Fig. 3b). The culture filtrate of B-RS3 was heated to destroy the antibiotics. Thereafter, it was mixed with *E. coli* harboring *ROMT9*. Culture filtrate from the second mixture was analyzed using HPLC. A new peak with a retention time different from that of resveratrol was observed (Fig. 3c). Mass spectrometry and proton NMR identified the synthesized compound to be pterostilbene. Approximately, 88.2% of the synthesized resveratrol during the first step was converted into pterostilbene in the second reaction.

Four other resveratrol derivatives (4-methoxyresveratrol, piceatannol, rhapontigenin, and isorhapontigenin) were also synthesized using the two-culture system. Each *E. coli* strain harboring the different modification genes for resveratrol was tested. *E. coli* strains individually harboring *HpaBC* and *SOMT* successfully converted resveratrol from the first cells into piceatannol (Fig. 3e) and 4-methoxyresveratrol (Fig. 3d), respectively. *E. coli* strains that harbored *SOMT2* and *HpaBC* synthesized rhapontigenin (Fig. 3f), while the ones with *ROMT15* and *HpaBC* synthesized isorhapontigenin (Fig. 3g). The conversion rates for 4-methoxy resveratrol, piceatannol, rhapontigenin, and isorhapontigenin from resveratrol were 72.6%, 71.1%, 22.8%, and 63.5%, respectively. Similar to pterostilbene, structures of the synthesized compounds were determined using proton NMR (Materials and methods).

Anti-inflammatory activity of resveratrol derivatives

Anti-inflammatory effects of resveratrol and five resveratrol derivatives were tested. Relative cell viability and the level of released cytokines were quantitatively measured using protein quantification methods and ELISA after LPS activation with or without resveratrol and resveratrol derivatives (Fig. 4). While resveratrol, 4-methoxyresveratrol, pterostilbene, and isorhapontigenin showed negligible toxicity, rhapontigenin and piceatannol elicited the toxic effect after treatment for 18 h. Cytokine (IL-6 and TNF-α) levels after LPS treatment in the presence of resveratrol derivatives are shown in Fig. 4b, c. Resveratrol and its derivatives successfully reduced the level of both cytokines in RAW264.7 cells. Levels of IL-6 were reduced to 13.8 ± 2.3, 8.3 ± 2.6, 0.5 ± 0.1, 5.9 ± 3.8, 9.6 ± 1.9, and 3.9 ± 0.3 ng/ml upon treatment with resveratrol, 4-methoxyresveratrol, pterostilbene, isorhapontigenin, rhapontigenin, and piceatannol, respectively, compared to the control (80.9 ± 17.8 ng/ml) (Fig. 4b). Previous studies have reported that resveratrol (20–25 μM) reduces the level of IL-6 by 3 ~ 8-folds in LPS-activated RAW264.7 cells (1 μg/mL of LPS) [52, 53]. In the current study, resveratrol (10 μM) induced a fivefold lowering in the IL-6 level, compared to the control; the observation was consistent with the previous studies [52, 53]. TNF-α also showed a significant decrease compared to control upon treatment with resveratrol and its derivatives (Fig. 4c). In particular, pterostilbene exhibited an approximate threefold lowering in the TNF-α release from LPS-activated RAW264.7 cells without any toxicity. Taken together, pterostilbene evidently enhanced the anti-inflammatory effects for macrophages, compared to resveratrol. Considering that methylation of the hydroxyl group at 3` and

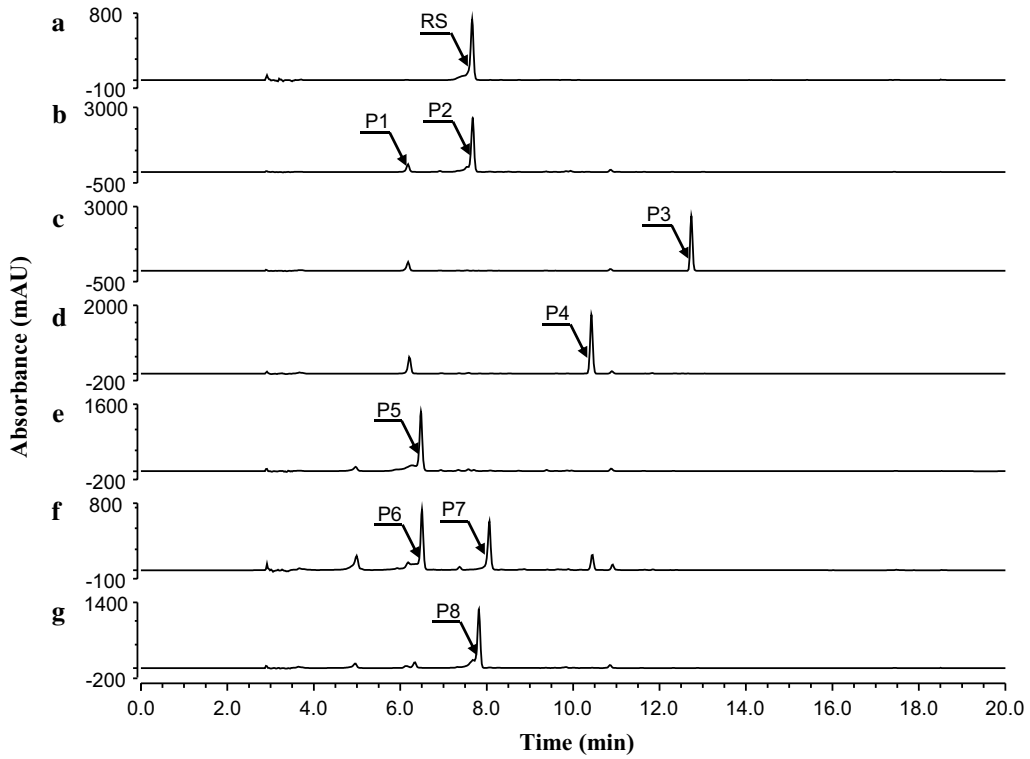

Fig. 3 High performance liquid chromatography (HPLC) analysis of the reaction products after modification of resveratrol. **a** HPLC profile of standard resveratrol. **b** HPLC profile of reaction products from B-RS3 strain. P1 and P2 were identified to be *p*-coumaric acid and resveratrol, respectively. **c** HPLC profile of reaction products from BL21 strain harboring *ROMT9*. P3 was determined to be pterostilbene. **d** HPLC profile of reaction products from BL21 strain harboring *SOMT2*. P4 was identified to be 4-methoxyresveratrol. **e** HPLC profile of reaction products from BL21 strain harboring *HpaBC*. P5 was identified to be piceatannol. **f** HPLC profile of reaction products from BL21 strain harboring *SOMT2* and *HpaBC*. P6 and P7 were determined to be piceatannol and rhapontigenin, respectively. **g** HPLC profile of reaction products from BL21 strain harboring *ROMT15* and *HpaBC*. P8 was determined to be isorhapontigenin. All the reaction products were purified and their structures were determined using nuclear magnetic resonance spectroscopy (See Materials and methods)

5` positions in resveratrol shows increased bioavailability and reduced metabolism, pterostilbene might have a significant anti-inflammatory effect in vivo, compared to resveratrol [54–56].

In this study resveratrol and five resveratrol derivatives were synthesized and evaluated for their anti-inflammatory activity. Pterostilbene showed the highest yield and the best anti-inflammatory activity. Final yield of pterostilbene can be enhanced if the initial cell concentration, culture time, and/or the copy number of resveratrol modification genes are optimized.

Majority of the biosynthesis studies that focused on the production of a target compound, employed techniques to engineer the host strains. This study not only emphasized on the biosynthesis of diverse derivatives but also attempted to evaluate their biological activity. Synthesis of target compounds with known biological and chemical importance in a biological system is of significant importance. However, at times synthesis of diverse compounds and screening or evaluation of their derivatives might be more critical and prerequisite compared to exploration of strategies to enhance the productivity.

Conclusion

Resveratrol is plant-based phenolic compound and resveratrol derivatives possess anti-inflammatory, anticancer, and antioxidant properties. This study utilized a bacterial host to synthesize resveratrol itself and five resveratrol derivatives. A two-culture system was used for this purpose. The first cell synthesized resveratrol while the second cell comprising resveratrol modification genes synthesized the resveratrol derivatives, namely isorhapontigenin, pterostilbene, 4-methoxyresveratrol, piceatannol, and rhapontigenin. The yield for resveratrol was 89.8 mg/L, while the conversion rate for the resveratrol derivatives was 88.2% to 22.9%. Anti-inflammatory activity of the synthesized resveratrol derivatives was evaluated and pterostilbene was shown the best anti-inflammatory activity. Successful synthesis of resveratrol derivatives with a satisfactory yield using a bacterial

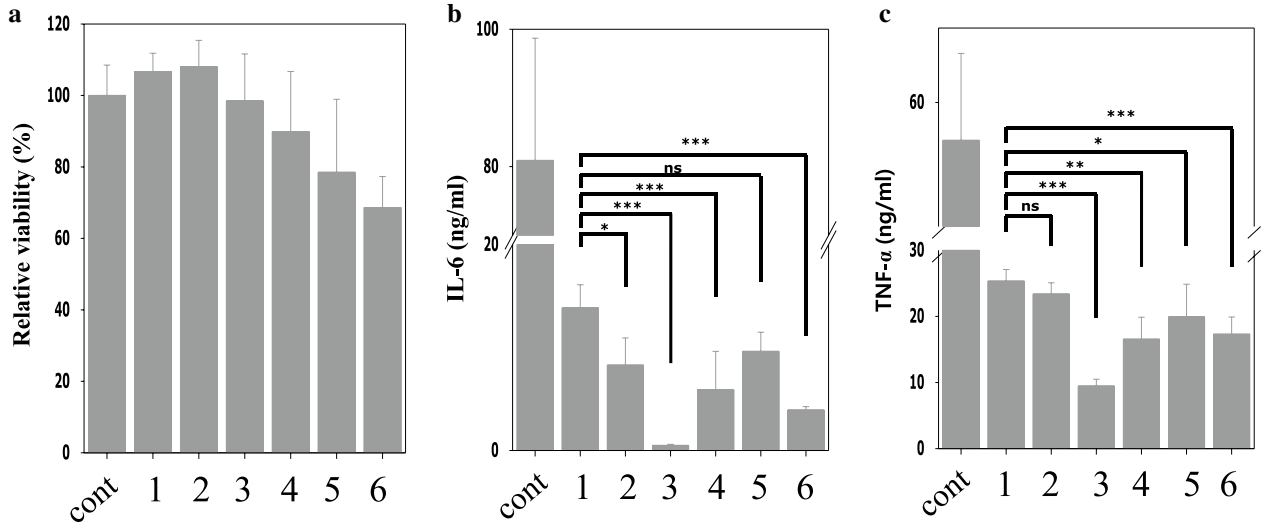

Fig. 4 Anti-inflammatory activity of resveratrol derivatives. **a** Relative cell viability after treatment with lipopolysaccharide (LPS) and resveratrol derivatives. **b** Level of released interleukin 6 (IL-6) after LPS treatment in the presence of resveratrol derivatives. **c** Level of tumor necrosis factor α (TNF- α) after LPS treatment in the presence of resveratrol derivatives. Control (Cont), no treatment; 1, resveratrol; 2, 4-methoxyresveratrol; 3, pterostilbene; 4, isorhapontigenin; 5, rhapontigenin; 6, piceatannol. All the compounds were used at a concentration of 10 μM. ns, *, **, and *** indicates not significant, $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

host cloned with resveratrol modification genes would indeed serve as a good source for synthesis of resveratrol derivatives.

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Authors' contributions
HM and JHA designed the experiments. YC, HLL, JS, YL, BGK and JHA performed the experiments and analyzed the data. YC, HLL, BGK, HM and JHA wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during the present study are included in this published article.

Declarations

Competing interests
The authors declare that they have no competing interests.

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