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Enzymatic glucosylation of citrus flavonoids to enhance their bioactivity and taste as new food additives

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Abstract

Cyclodextrin glucosyltransferase (CGTase) is commonly used to produce cyclodextrins but its utility in flavonoids modification is rarely found. In the present study, an enzymatic transformation of citrus flavonoids by applying CGTase as the catalyst was investigated to produce a number of novel polyglucosylated derivatives from the monoglucoside metabolites of citrus flavonoid diglycosides. Under the optimized conditions, the yield of glucosylated products achieved 80%. The water solubility and water partition coefficient of citrus flavonoids before and after the structural modification were compared. The results revealed that the water solubility of the flavonoids modified by glucosylation was significantly increased while the oil-water partition coefficient was just slightly reduced. Moreover, after glucosylation, the products have good anti-inflammatory and antioxidant effects. Interestingly, the taste property (bitterness and sweetness) of the glucosylated products was ameliorated. An evaluation with a Taste-Sensing System showed that the taste of the glucosylated products was strongly correlated with the attached rhamnosyl group (bitter taste) and glucosyl group (sweet taste), indicating that the amelioration of taste of flavonoids could be mainly attributed to the addition of glycosyl groups. The present study demonstrated a novel molecular engineering approach for the enhancement of water solubility and amelioration of taste for some citrus flavonoids, which may have potential applications in the functional food industry.

Keywords: Citrus flavonoids, Glucosylation, Cyclodextrin glucosyltransferase, Enzymatic reaction, Food additives

1. Introduction

Citrus flavonoids are widely distributed in nature and usually exist in the form of glycosides such as glucosides, galactosides, rhamnosides, arabinosides and rutinosides[1]. These natural products play important roles in many biological processes[2]. Many studies showed that citrus flavonoids could act as antioxidants and have good antioxidant effects in the prevention of cancer, cardiovascular diseases, gastric ulcers, allergies and even antibacterial infections[3,4,5]. Some citrus flavonoids are able to improve the flavor of foods and beverages because the compounds have de-bittering or sweetening property and can be used as natural flavorings, such

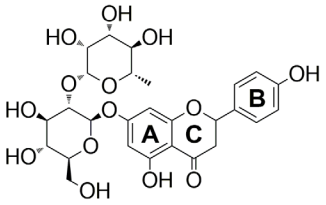
as those utilizing in alcohol, jam, natural food additives and health care products[6]. In recent years, citrus flavonoids have attracted great attention from food industry because the natural food additives are high-valued. The methodology development for the extraction of natural flavonoids in high purity from plants for further formulation into food and medicine to offer certain desirable functions has been an industrial demand[7].

Consumers more interest in healthy food as they want to eat healthy nowadays. Functional foods, for example probiotic coffee, attract health-conscious consumers. In food processing, taking the concept of food-as-medicine, some healthy ingredients and additives are usually formulated into food[8]; but interestingly, in most of the cases, people probably needs to sacrifice the taste because some natural additives like polyphenols[9], vitamins[10] and minerals[11] usually result unfavorable tastes such as bitterness. Therefore, the good taste (de-bitterness) and healthy flavor and improvers to make products palatable enough for consumers are demanding in industry[12]. The research and development of flavonoids as food additives are currently progressed rapidly and some functional beverages and foods containing flavonoids have been developed[13]. These types of food have good taste and also are easy to be preserved because flavonoids have good antibacterial activity[14]. It was also reported that homoeriodictyol and hesperetin can mask or block the bitter taste in food and enhance sweetness. Moreover, neodiosmin is effective in reducing bitterness in orange juice at threshold and suprathreshold levels of limonin[15,16,17]. It was also reported that phloretin can be used as the best bitterness masking agent for caffeine but neohesperidin dihydrochalcone (NHDC) is much less effective on blocking bitter taste[18]. Some healthy drinks are produced using blood orange juice as the raw material and formulated with functional flavonoids to promote metabolism, anti-infection, heatstroke prevention and thirst quenching[19].

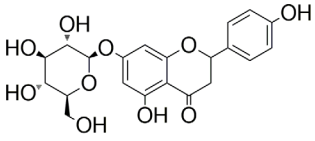
In recent years, citrus peel extracts have been highlighted on its potential bioactivity in promoting human health. The main bioactive compounds identified from citrus peels include hesperidin and naringin. Despite the citrus flavonoids are known to offer a variety of biological activities including anti-inflammatory, antiallergic and neuroprotective effects[20,21], citrus flavonoids encounter some major disadvantages, such as poor water solubility and low bioavailability, limiting their potential application in the food industry. To address these

problems, Fasinu and Yamada attempted to modify the structure of citrus flavonoids through biological or chemical methods[22]. It was reported that glycosylation or de-glycosylation are effective to improve the physiological property[23,24].

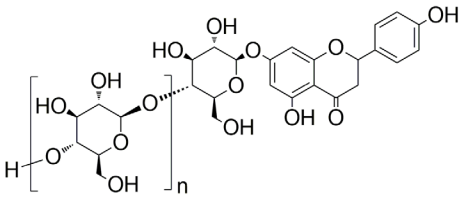
Structure modification of citrus flavonoids is a recognized approach to enhance their biofunctions and enzymatic approach is an effective method for industrial process[25], particularly using cyclodextrin glucosyltransferase (CGTase) as the catalyst[26,27,28,29,30,31,32,33,34]. In the present study, our primary objective is to modify the structure of citrus flavonoids in order to reduce their unfavorable properties including taste, poor water solubility and low bioavailability for industrial applications such as healthy food flavor and improvers. A number of uncommon flavonoid oligoglucosides (**Scheme 1**) were produced through the biological approach, which utilizes CGTase as the catalyst[35,36,37,38,39,40,41] to modify the structure of citrus flavanone (mono- or multi-glucosylated). The citrus flavanone glycosides including monoglucosides **(1a−6a)** and diglycosides **(1−6)** were selected for the structural engineering study. The water solubility, oil-water partition coefficient, cytotoxicity, anti-inflammatory and the antioxidant of these isolated glucosylation products were investigated. In addition, the taste and de-bitterness ability of the newly modified citrus flavonoids were evaluated with a Taste-Sensing System (electronic tongue).



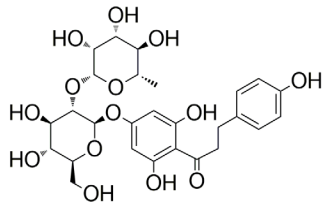
naringin (**1**)



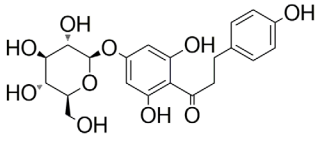
prunin (**1a**)



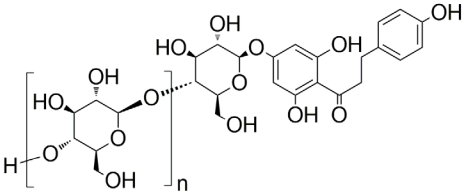
prunin-Gn (**1a-Gn**)



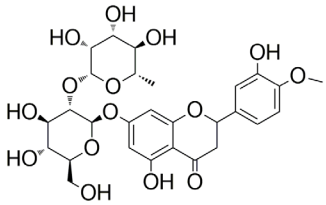
naringin dihydrochalcone
(Nar DC) (**2**)



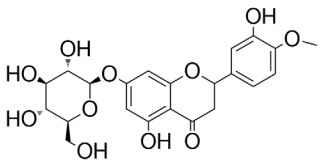
trilobatin (**2a**)



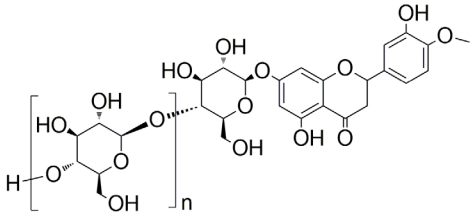
trilobatin-Gn (**2a-Gn**)



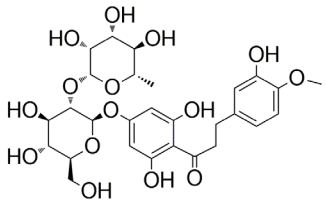
neohesperidin (**3**)



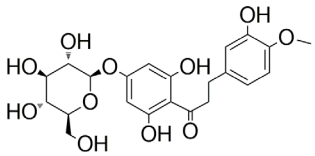
hesperetin-7-O-glucoside
(Hes-7-G) (**3a**)



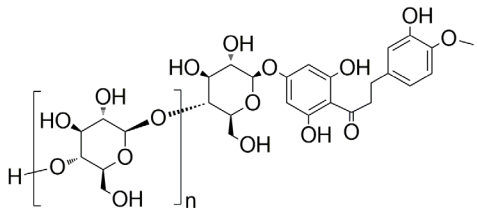
hesperetin-7-O-glucoside-Gn
(Hes-7-G-Gn) (**3a-Gn**)



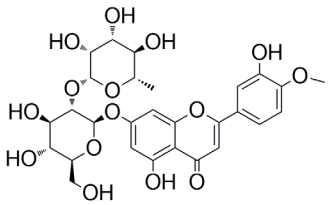
neohesperidin dihydrochalcone
(**NHDC**) (**4**)



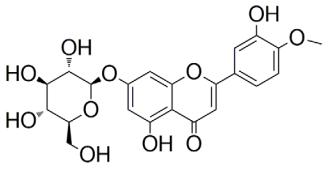
hesperetin dihydrochalcone
-7-O-glucoside
(**HDC-7-G**) (**4a**)



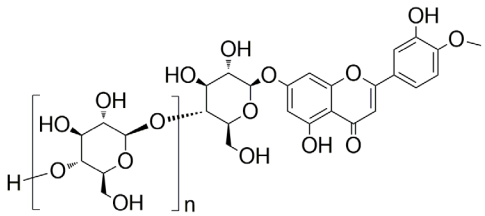
hesperetin dihydrochalcone
-7-O-glucoside-Gn
(**HDC-7-G-Gn**) (**4a-Gn**)



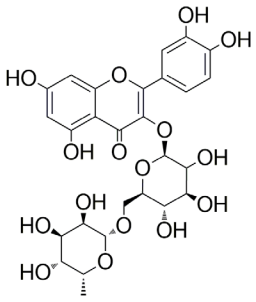
neodiosmin (**5**)



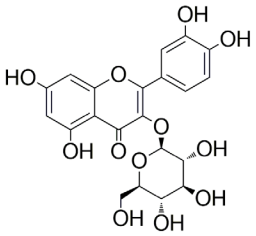
diosmetin-7-O-glucoside
(**Dio-7-G**) (**5a**)



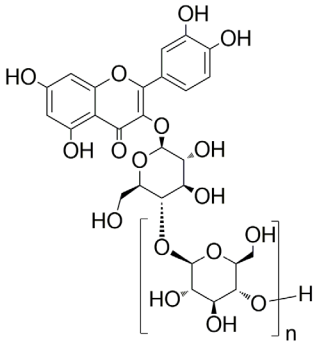
diosmetin-7-O-glucoside-Gn
(**Dio-7-G-Gn**) (**5a-Gn**)



rutin (**6**)



isoquercitrin
(**IQC**) (**6a**)



isoquercitrin-Gn
(**IQC-Gn**) (**6a-Gn**)

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Scheme 1. Chemical structures of flavonoid diglycosides **(1−6)**, monoglucosides **(1a−6a)** and polyglucosides **(1a-G_n−6a-G_n)**.

2. Experimental

2.1 Chemical reagents and materials. The dihydrochalcones standards including naringin dihydrochalcone **(2)**, neohesperedin dihydrochalcone **(4)** and the flavanones standards includingnaringin **(1)**, neohesperidin **(3)**, neodiosmin **(5)**, rutin **(6)** were purchased from Sigma. Octanol was purchased from Sigma. HPLC grade acetonitrile and acetic acid were purchased from Oceanpak (Hanover Park, IL). Silver chloride and tartaric acid were purchased from Aladdin. All other used chemicals and reagents were of analytical grade and were used without further purification. CGTase (90 U/mL) was provided by Jinjunkang Inc., Guangdong, China.

2.2 Enzymatic synthesis of monoglucosides: trilobatin, prunin, Hes-7-G, HDC-7-G, Dio-7-G and isoquercitrin

The enzymatic synthetic methods utilized in this study were followed the reported procedures with slight modifications[42]. Briefly, 200 mg citrus flavonoid glycosides (naringin **(1)**, naringin dihydrochalcone **(2)**, neohesperidin **(3)**, neohesperidin dihydrochalcone **(4)**, neodiosmin **(5)**, and rutin **(6)**) were dissolved in 100 mL of deionized water or a mixed solvent. After ultrasound for 10 min, the mixture was placed in a water bath oscillator with constant temperature at 60 °C for 30 min. After the substrate is completely dissolved or a well-dispersed suspension is formed, the immobilized α-L-rhamnosidase (100 mg) was added to the solution. Then, the oscillation frequency was set at 150 r/min for 2-5 h reaction at 60 °C. TLC was used to monitor the reaction process. After the reaction was completed, hot filtration was carried out. The solid enzyme rhamnosidase was separated and rinsed with warm water (40 °C) and it was then recycled in the next reaction cycle. The filtrate collected was cooled at 4 °C in a refrigerator overnight. The solid monoglucoside products **(1a−6a)** were separated. The crude products were recrystallized with 80% ethanol to obtain pure monoglucoside products. The isolated yield and detailed product characterization and purity were given in Supporting Information.

2.3 Enzymatic synthesis of glucosylated flavonoids by CGTase

The enzymatic synthetic methods were referred to the reported procedures[43]. 1 g of monoglucoside (prunin **(1a)**, or trilobatin **(2a)**, or hesperetin-7-O-glucoside **(3a)**, or hesperetin dihydrochalcone-7-O-glucoside **(4a)**, or diosmetin-7-O-glucoside **(5a)**, or isoquercitrin **(6a)**) were dissolved in deionized water (or in a pH 8 phosphate buffer for the substrate of hesperetin-7-O-glucoside **(3a)**, diosmetin-7-O-glucoside **(5a)**, and isoquercitrin **(6a)**) by sonication. Then, to this solution, 20 g of dextrin were added. The mixture was then incubated in a water bath shaker with constant temperature at 45 °C. Finally, cyclodextrin glucosyltransferase (300 U/L) was added to the mixture and then the oscillation frequency of sonication was set at 120 r/min to facilitate the reaction. The progress of glucosylation reaction was monitored by HPLC every 30 min until the glucosylated products had no further increase. After reaction, the mixture was extracted with a solution of *n*-butanol saturated with ultrapure water (for 2 times) to remove most of unreacted dextrin starting and enzyme residues. Then, the aqueous mixture collected was purified by macro-porous resin to afford the multi-glucosylated ($\mathbf{G_n}$) products. The major monoglucosylated product ($\mathbf{G_1}$) was purified and isolated with flash silica gel column chromatography. The isolated yield and detailed product characterization and purity were given in Supporting Information.

2.3.1 Kinetic study for CGTase activity in the glucosylation reaction

For kinetic analysis of CGTase in catalyzing the glucosylation reaction with four representative flavonoid substrates (prunin (**1a**), trilobatin (**2a**), Hes-7-G (**3a**), IQC (**6a**)), 30 U enzymes were added to de-ionized water and 1 g maltodextrin in a final volume of 100 mL. The concentration of the flavonoid substrate was ranged from 7.5 to 960 mM, and after 30 min incubation at 25 °C, the reaction was stopped by heating to 100 °C for 5 min. The reaction mixture was analyzed by HPLC. Kinetic parameters V_{max} and K_M were calculated by doing Michalis-Menten nonlinear curve fitting in GraphPad Prism 8.0.

2.4 Water solubility analysis and oil-water partition coefficient assays

The method for water solubility analysis of the compounds was followed the reported literature with slight modifications[44]. The oil-water partition coefficient assay was performed

according to the reported method with some modifications[45]. The detailed experimental procedures were given in Supporting Information.

2.5 MTT cytotoxic activity experiment of glucosylated flavonoids

Cytotoxicity was determined using the tetrazoliumbased colorimetric assay (MTT test)[46]. The new compounds **(1a-G_1−6a-G_1)** were dissolved in dimethyl sulfoxide (DMSO) and diluted in the culture fluid to get various concentrations (8, 16, 32, 64, 128, 256 μM). The cells were treated with target compounds subsequently and incubated in a 37 °C, 5% CO_2 incubator for 24 h. Then 100 μL MTT (0.5 mg/mL) was added to the plate and incubated for 4 h. The medium was removed, and 100 μL DMSO was added to each microwell to solubilize the formazan produced from MTT during incubation with cell lines. The absorbance was determined at 570 nm with a microplate reader.

2.6 NO inhibitory assay

Anti-inflammatory effects of the glucosylated flavonoids were evaluated via the measurement of the accumulated NO in the culture supernatant induced by lipopolysaccharide (LPS) in macrophages cell line RAW 264.7 utilizing Griess reaction described in the literature[47].

2.7 Antioxidant assay

The FRAP assay was performed according to the reported method with some modifications[48]. The FRAP reagent was made by the addition of 300 mM acetate buffer (pH 3.6) with 10 mM TPTZ (in 40 mM HCl) and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1. Sample (20 μL) and distilled water (180 μL) were added to 3 mL of working FRAP reagent and kept at 37 °C for 4 min. The absorbance was measured at 593 nm, and the FRAP value was calculated as mM of Fe^{2+} equivalent/g DW of a sample using the standard graph of $FeSO_4$ with a linearity range of 0.1 to 2.5 mM.

DPPH radical scavenging activity was evaluated by measuring the DPPH radical scavenging utilizing the DPPH assay as previously described[49]. Briefly, DPPH was prepared

in methanol at the concentration of 0.1 mM, and 150 μL of this solution was mixed with 50 μL of the test sample (dissolved in methanol) at the concentration of 50 μM and 100 μM. These solutions were mixed and incubated at 25 °C in the dark for 30 min. Finally, the OD value was determined at 517 nm using a plate reader. The percentage of DPPH scavenging was calculated with the formula[50]: DPPH scavenging (%) = [(Control absorbance–sample or standard absorbance)]/Control absorbance] × 100.

2.8 Taste assessment of the glucosylated flavonoids with a taste evaluation system

The electrical taste profile of flavonoids was determined by the Taste-Sensing System SA-402B. The system consists of six sensors: sweetness (GL1), bitterness (C00) and astringency (AE1), saltiness (CT0), sourness (CA0) and umami (AAE). Each sensor electrode is immobilized with a lipid/polymer membrane[51,52]. The sensors respond to each basic taste according to the concentrations and the combination of the lipid and the plasticizer. The measurement and cleaning procedures were followed the reported study[53,54]. The reference and sensor electrodes include Ag/AgCl electrodes and an internal solution containing 3.33 M KCl and saturated AgCl. The voltage difference between sensor electrode and reference electrode was tested. The stability and accuracy of the sensor were examined using a mixture of reference solution (30 mM KCl and 0.3 mM tartaric acid)[55].

2.8.1 Measurement setup for the compound with a Taste-Sensing System

The sensor electrodes were immersed in a reference solution of 30 mM KCl and 0.3 mM tartaric acid, which mimics human saliva with almost no taste. The measurement cycle begins with three washing steps in the washing solution. The sample was then analyzed for 30 s, followed by two short washing steps for 3 s, and aftertaste detection for 30 s. Each sample was measured 5 times in random order, always starting with the reference solution to monitor the sensor response. The best range of sensor potential response was measured with the series of Trilobatin (**2a**) solutions prepared with the concentrations of 0.01, 0.03, 0.06, 0.1, 0.3, 0.6, 1 mg/mL in a 10 mM KCl solution. The appropriate compound concentration was determined to

be 40 μg/mL. The taste values of sweetness and bitterness of the compounds (**1**−**6, 1a**−**6a** and (**1a-G_n**−**6a-G_n**)) at 40 μg/mL were measured independently.

3. Results and discussion

3.1 Preparation of glucosylated flavonoids using CGTase as the catalyst

In the enzymatic reactions, six flavonoid monoglucosides (**1a**−**6a**) were prepared and isolated from the corresponding diglycosides including naringin **(1)**, Nar DC **(2)**, neohesperidin **(3)**, NHDC **(4)**, neodiosmin **(5)** and rutin **(6)** using an immobilized α-L-rhamnosidase (Imm-α-L-Rha) as the catalyst for hydrolysis reactions according to the reported methodology[42]. This enzymatic method applying CGTase (cyclodextrin glucosyltransferase) as the catalyst for the glucosylation reaction of citrus flavonoid monoglucosides **(1a**−**6a)** was then investigated. Six flavonoid monoglucosides **(1a**−**6a)** were used as the substrates for the glucosylation study, in which the enzymatic reaction conditions were optimized with trilobatin **(2a)** as a model substrate. The glucosylated product is a mixture that has different number of glucosyl groups added. The glucosylated products were denoted as $\mathbf{G_n}$ and the $\mathbf{G_1}$ analogue was separated by HPLC. These pure monoglucosylated products ($\mathbf{G_1}$ represents only one glucosyl group was added), **1a-G_1**−**6a-G_1**, were further characterized by ^{1}H NMR, ^{13}C NMR and ESI-MS to confirm their structures.

3.2 The optimization of enzymatic glucosylation reaction

Trilobatin **(2a)** was selected as the model substrate in the optimization of the glucosylation reactions because it is more water-soluble than other compounds and can be completely dissolved in phosphate buffers at lower temperature compared with other analogues. Glycosyl donor is an important raw material for glucosylation reaction. Due to CGTase is highly selective in glucosylations, the selection of glycosyl donor may also play a critical role in the reaction. To study the effect of different glycosyl donors, the reaction conditions were fixed under the following conditions: trilobatin was 0.1 g (0.22 mmol/L) and glycosyl donor amount was 1 g, CGTase was 300 U/L, reaction temperature was at 45 °C and the reaction was conducted in deionized water for 10 h. Under these conditions, 8 different glycosyl donors including glucose, sucrose, starch, maltodextrin, α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), lactose and xylan

were examined. The conversions of glycosyl donors in the reaction were shown in **Figure 1A**. Maltodextrin, α-CD, β-CD and starch gave much higher conversion (66-76%) than glucose, sucrose, lactose and xylan, which have only 1-8% conversion. The results may indicate that β-CD is the best substrate in the CGTase catalyzed glucosylation reaction among the glycosyl donors tested. However, β-CD is much more expensive than maltodextrin and starch and thus it may not be the best choice for industrial applications. On the contrary, maltodextrin has better water-soluble than β-CD and is a low-cost glycosyl donor. More importantly, maltodextrin is largely available from commercial. We therefore selected maltodextrin for the subsequent experiments.

The pH effect on the CGTase catalyzed glucosylation reaction was investigated. In general, pH conditions may have significant impacts on the bioactivity of enzymes. The tolerance of enzymes from different strains to the acid-base environment is also different. Only under the suitable pH condition, the activity of the enzyme can be maximized. In the glucosylation, we found that the water solubility of citrus flavonoids is extremely poor and it can only show better solubility under alkaline conditions. Therefore, it is essential to study the alkali tolerance of CGTase. The experiments were conducted in a phosphate buffer at different pH conditions with trilobatin (0.1 g, 0.22 mmol/L) as the substrate, maltodextrin (1 g) as the glycosyl donor, and CGTase 300 U/L at 45 °C for 10 h. The effects of pH from 4 to 13 were compared in terms of maltodextrin conversion in the reaction. As shown in **Figure 1B**, CGTase has a wide range of pH tolerance and exhibits good catalytic activity in the range of pH 5 to 12. However, under high acidic (pH < 5) or high alkaline (pH > 13) conditions, the enzymatic activity of CGTase is significantly declined or even inactive. Due to citrus flavonoids having higher solubility in alkali solution than in acidic medium, pH=10－12 was thus selected as the optimal condition for CGTase catalyzed glucosylation in the following experiments.

The effect of temperature on the CGTase catalyzed glucosylation was also investigated. The experiments were conducted with trilobatin **(2a)** (0.1 g, 0.22 mmol/L) as the substrate, maltodextrin (1 g) as the glycosyl donor, and CGTase 300 U/L at different temperature conditions for 10 h. The effects of temperature ranged from 20~80 °C in the glucosylation reaction were determined by the conversion of maltodextrin. As shown in **Figure 1C**, it was found that CGTase

was active and gave comparable conversions (64~67%) at 20~50 °C. However, when the reaction temperature was conducted at 70 °C or higher, CGTase activity was found decreased significantly (< 36% conversion). In addition, since citrus flavonoids have better solubility at 50 °C, this temperature condition is thus applied for further experiments.

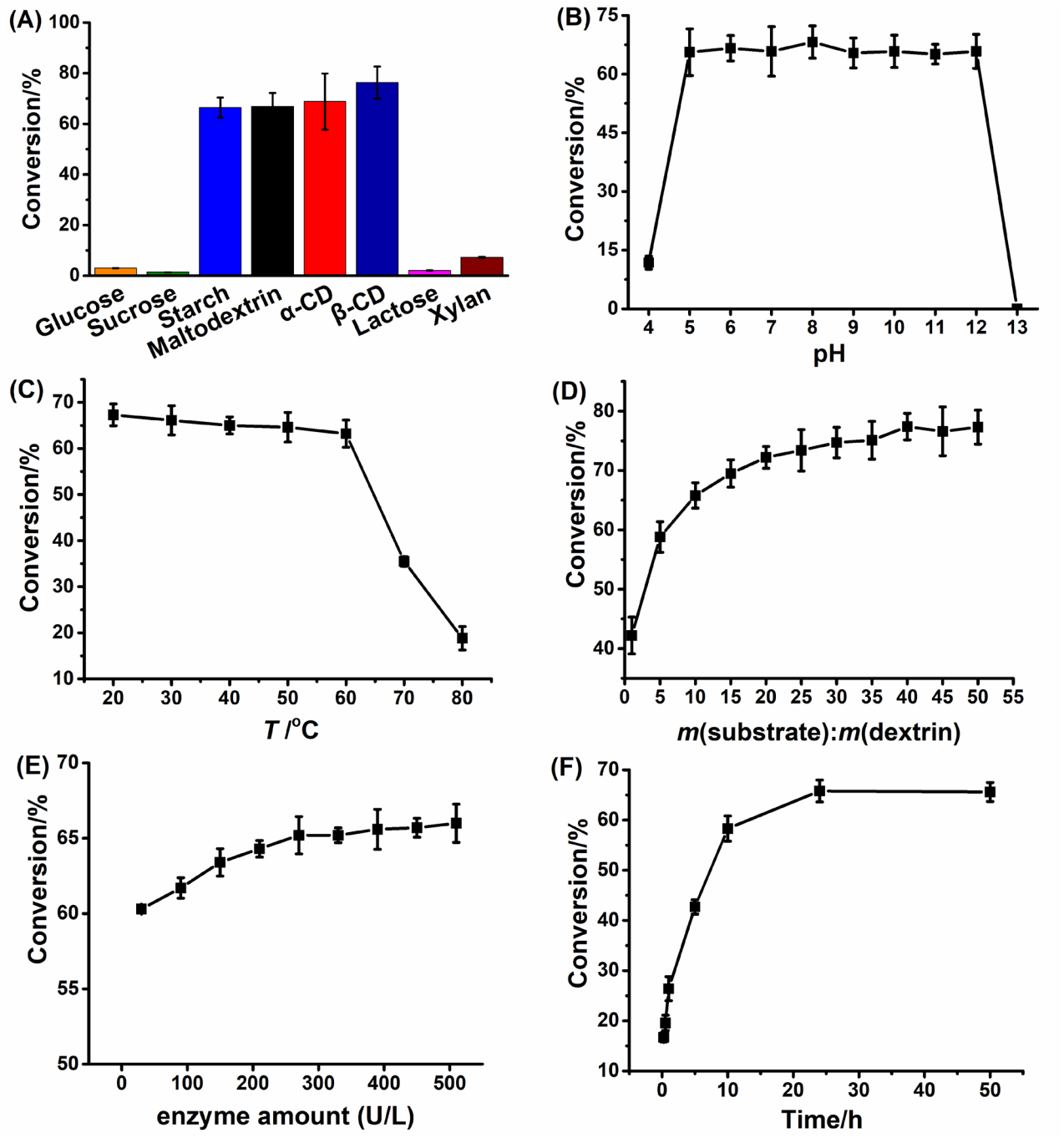


Figure 1. Optimization study for the for the enzymatic glucosylation reaction with trilobatin **(2a)** as a model substrate: (A) the effects of glycosyl donors; (B) the effects of pH; (C) the effects of temperature; (D) the effects of substrates ratio; (E) the effects of catalyst loading in the reaction and (F) the effects of time.

The effect of substrate to glycosyl donor ratio (mass ratio) on the CGTase catalyzed glucosylation was investigated. The experiments were conducted in 100 mL of deionized water with CGTase 300 U/L at 50 °C for 10 h with a fixed amount of trilobatin substrate (0.1 g) and varying the amount of glycosyl donor (maltodextrin = 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 g, respectively). The ratio effect of the glucosylation reaction in terms of maltodextrin conversion was compared. The results shown in **Figure 1D** reveal that when increasing the mass ratio of maltodextrin/trilobatin in the reaction, the conversion was increased gradually from 42% to 77%. When the ratio was higher than 40, the conversion was found reaching a plateau. Therefore, the mass ratio of maltodextrin to trilobatin **(2a)** at 40 was the optimal value.

The loading of enzyme for the glucosylation reaction was also optimized. The reactions were conducted with a fixed amount of trilobatin **(2a)** (0.1 g) and maltodextrin (1 g) in de-ionized water at 50 °C for 10 h by varying the amount of CGTase. The effect with respect to the conversion of maltodextrin was shown in **Figure 1E**. It was found that the conversion increased gradually with the increase of enzyme loading and a plateau of conversion was found at 270 U/L, which could be the optimal loading of CGTase for the reaction.

The effect of reaction time on glucosylation reaction was investigated. In the reactions, the amount of trilobatin **(2a)** (0.1 g), maltodextrin (1 g), and CGTase (300 U/L) in de-ionized water was fixed and the temperature was 50 °C. The reaction time of 0.25, 0.5, 1, 5, 10, 24 and 50 h were evaluated for maltodextrin conversion. As shown in **Figure 1F**, the conversion increased gradually from 41% to 77%, indicating that the optimal reaction time was 24 h.

Through the above reaction optimization for CGTase catalyzed glucosylation of trilobatin **(2a)**, it was found that the trilobatin **(2a)** conversion was able to reach 80% under the buffered conditions at pH 8, maltodextrin 4 g, trilobatin 0.1 g (0.22 mmol), CGTase 300 U/L, 20 °C, 120 r/min for 24 h. Moreover, under the optimized conditions, the high conversions for other citrus flavonoids including prunin **(1a)** = 79%, hesperetin-7-O-glucoside **(3a)** = 75%, hesperetin dihydrochalcone-7-O-glucoside **(4a)** = 80%, diosmetin-7-O-glucoside **(5a)** = 81% and isoquercitrin **(6a)** = 72% were also achieved.

The enzyme kinetics of CGTase in catalyzing the glucosylation reaction with four representative substrates was also investigated. The initial rate of the reaction was measured over a

range of substrate concentrations (7.5 to 960 mM). It was found that the initial reaction rate increased when the substrate concentration increased as shown (**Figure 2**). Based on the Michaelis–Menten kinetic model of a single-substrate reaction, the kinetic parameters V_{max}, K_M, k_{cat}, and k_{cat}/K_M were determined and listed in **Table 1**. Among the substrates tested, trilobatin exhibited the highest V_{max} and k_{cat}, and its K_M was lowest. Therefore, the k_{cat}/K_M value for trilobatin is the largest, which may indicate that trilobatin is a suitable substrate for the CGTase-catalyzed glucosylation reaction under the optimized conditions.

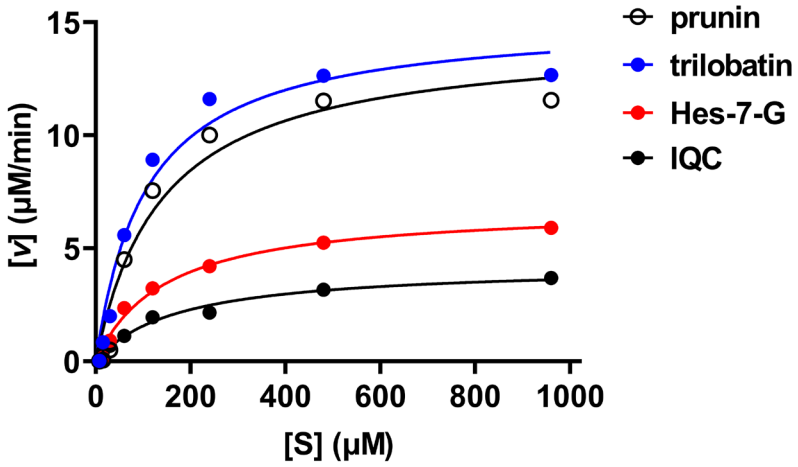


Figure 2. The saturation curves for CGTase catalyzing the glucosylation reaction based on Michaelis-Menten equation.

Table 1. Kinetic parameters of CGTase towards different flavonoids in the glucosylation reaction.

Substrate	V_{max} (μmol·L^{-1}·min^{-1})	K_M (μmol·L^{-1})	k_{cat} (μmol·U^{-1}·s^{-1})	k_{cat}/K_M (L·U^{-1}·s^{-1})
trilobatin (**2a**)	15.2	106.1	8.44×10^{-4}	7.92×10^{-6}
prunin (**1a**)	14.4	139.9	7.98×10^{-4}	5.72×10^{-6}
Hes-7-G (**3a**)	6.9	145.4	3.81×10^{-4}	2.61×10^{-6}
IQC (**6a**)	4.3	170.7	2.36×10^{-4}	1.41×10^{-6}

3.3 HPLC analysis of the glucosylation products with trilobatin (2a) as the model substrate

CGTase is a ubiquitous group of enzymes that catalyze the formation of glycosidic linkages by transferring a sugar residue from a donor to an acceptor. This mechanism requires the presence of a correctly positioned nucleophile (Asp or Glu residue) at the catalytic center, which mediates the formation of a glycosyl-enzyme intermediate in the first part of the reaction, resulting in a first anomeric inversion. After that, the activated receptor substrate attacks the glycosyl-enzyme intermediate and results the formation of the product[56]. This reaction process is inferred according to our results observed in glucosylation of trilobatin **(2a)** with CGTase as the catalyst. In the reaction, maltodextrin was hydrolyzed by CGTase into glucosyl, which was then transferred to a trilobatin to produce trilobatin-G_1 **(2a-G_1)**. At this stage of the reaction, there are two the substrates including trilobatin **(2a)** and trilobatin-G_1 **(2a-G_1)** are available in the reaction system. As CGTase continues to catalyze the glucosylation of substrates, trilobatin-G_1 **(2a-G_1)**, trilobatin-G_2 **(2a-G_2)** and a series of multi-glucosylated products **(2a-G_n)** were formed. The multi-glucosylated products **(2a-G_n)** can be determined via HPLC analysis as shown in **Figure 3A**. The reaction was monitored by HPLC from 5 min to 10 h (shown in **Figure S46**). It was found that the multi-glucosylated products were increased gradually. The composition (**G_1**−**G_n**) of trilobatin glucosylates produced was shown in **Table S1**. For a 10 h reaction, the monoglucosylated analogue **(2a-G_1)** was found to be the major product. The exactly number of glucosyl group (**G**) transferred to trilobatin **(2a)** in the reaction were determined with mass spectroscopy (ESI-MS) (**Figure 3B**). Under the optimized conditions, the six monoglucosides **(1a**−**6a)** were carried out for the glucosylation reaction with maltodextrin as the glycosyl donor. The results were shown in **Table S2**. From HPLC analysis, the monoglucosylated analogues (**G_1**) **(1a-G_1**−**6a-G_1)** were the major product in the glucosylation reaction with about 14-20.9%. All these multi-glucosylated analogues were separated by HPLC for bioassays and taste evaluation study (sweetness and bitterness).

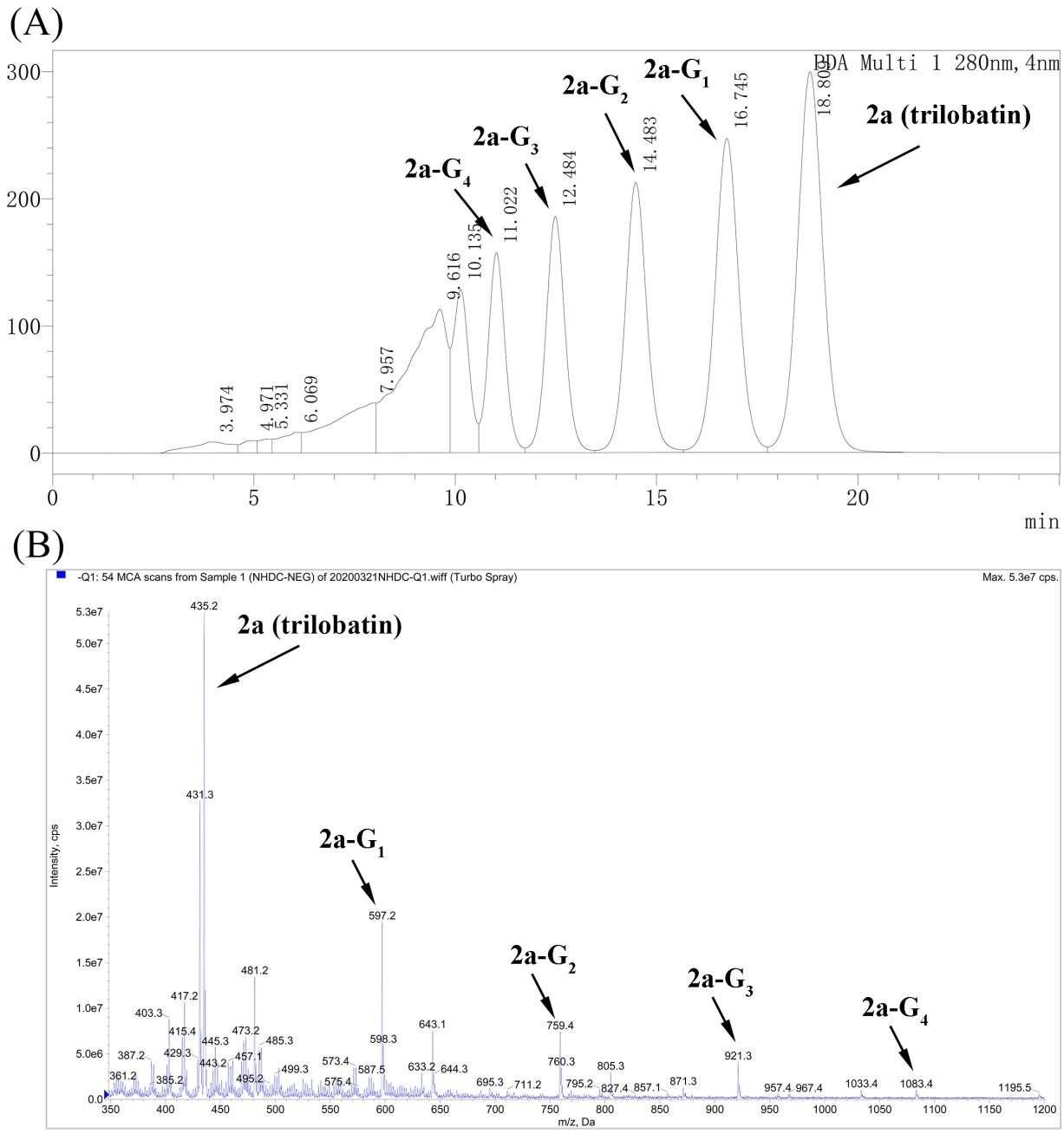


Figure 3. (A) HPLC analysis of the glucosylation reaction product with trilobatin (**2a)** as the model substrate for 10 h reaction and (B) mass spectroscopic characterization of the **2a** and its glucosylates **(2a, 2a-G$_{1-4}$)** from the reaction mixture.

3.4 Comparison of water solubility of diglycosides flavonoid (1－6), monoglucosides flavonoid (1a－6a) and glucosylated flavonoids (1a-G$_1$－6a-G$_1$ and 1a-G$_n$－6a-G$_n$)

The UV-vis absorbance of a series of standard solutions (2－8 μg/mL) of flavonoid diglycosides **(1－6)** and monoglucosides **(1a－6a)** was measured by UV-vis spectrophotometry.

The standard curves (optical density (OD) versus concentration) were plotted and the corresponding linear regression equations were shown as shown in **Figure S44**. The compounds exhibited excellent linear relationship within the concentration range measured. The saturation solubility of compounds **1**−**6**, **1a**−**6a** and **1a-G$_1$**−**6a-G$_1$** in ultrapure water were measured at 25 °C. The results were shown in **Table 2**. The solubility was found improved significantly after the addition of glycosyl group. The solubility of the compound was found generally in the following order: **1**−**6 < 1a**−**6a < (1a-G$_1$**−**6a-G$_1$)**. By comparing the solubility enhancement (in terms of solubility ratio), it was found that the glycosyl group introduced to the flavonoid glycosides would enhance the solubility, which is good for industrial food formulation process.

Table 2. Saturated solubility of compounds in ultrapure water at 25 °C.

Compound	Solubility (μM)	Solubility enhancement [a]
1	393.43 ± 2.2	1.0
1a	1225.7 ± 15.6	3.1
1a-G$_1$	1100.86 ± 20.1	2.8
2	778.10 ± 10.3	1.0
2a	1870.04 ± 56.2	2.4
2a-G$_1$	2238.42 ± 102.1	2.9
3	126.03 ± 6.1	1.0
3a	247.99 ± 15.6	2
3a-G$_1$	514.23 ± 16.4.2	4.1
4	430.08 ± 22.1	1.0
4a	753.41 ± 36.8	1.8
4a-G$_1$	1394.46 ± 143.6	3.2
5	7.40 ± 0.9	1.0
5a	14.50 ± 1.2	2
6	41.30 ± 3.5	1.0
6a	73.36 ± 6.8	1.8

[a] The ratio of the water solubility before and after glucosylated modification.

3.5 Comparison of oil-water partition coefficient for diglycosides flavonoid (1 − 6), monoglucosides flavonoid (1a−6a) and glucosylated flavonoids (1a-G_n−6a-G_n)

The oil-water partition coefficient $P_{o/w}$ is an important parameter to indicate the hydrophilicity or lipophilicity of a compound. The log $P_{o/w}$ value is the logarithm of the concentration ratio of the compound when the *n*-octanol organic phase and the aqueous phase reach equilibrium. The relationship of the concentration of the compound in aqueous phase c_w and *n*-octanol c_o is represented by the equation: log $P_{o/w}$ = log c_o/c_w. The smaller the log $P_{o/w}$ value, the stronger the hydrophilicity would be; and the larger the log $P_{o/w}$, the stronger the lipophilicity of the compound would be. The log $P_{o/w}$ value is a key factor in determining the pharmacokinetic parameters. When the value of log $P_{o/w}$ is small, the fat solubility of the compound is too poor to pass through the lipid, meaning that the drug cannot be absorbed in the intestinal tract. On the contrary, when the value of log $P_{o/w}$ is large, the compound is too water-soluble to penetrate the biofilm. The most suitable oil-water partition coefficient as a drug is $-1 < \log P_{o/w} < 2$.[57] The drug needs to have an appropriate oil-water partition coefficient in order to complete the transport (water-soluble) in the body and the diffusion of the biofilm (lipid-soluble) to the site where it binds to the receptor to produce a drug effect. The oil-water partition coefficients of the compounds were determined and shown in **Table S3**. We found that the log $P_{o/w}$ calculated is in the range of 0-1, which meets the requirements of the oil-water partition coefficient of the drug ($-1 < \log P_{o/w} < 2$). Moreover, after the removal of rhamnose groups and the addition of glucosyl groups were able to increase the hydrophilicity of the diglycosides flavonoids **(1 − 6)**. The alteration of their hydrophilicity characters through glucosylation could be able to improve their bioavailability.

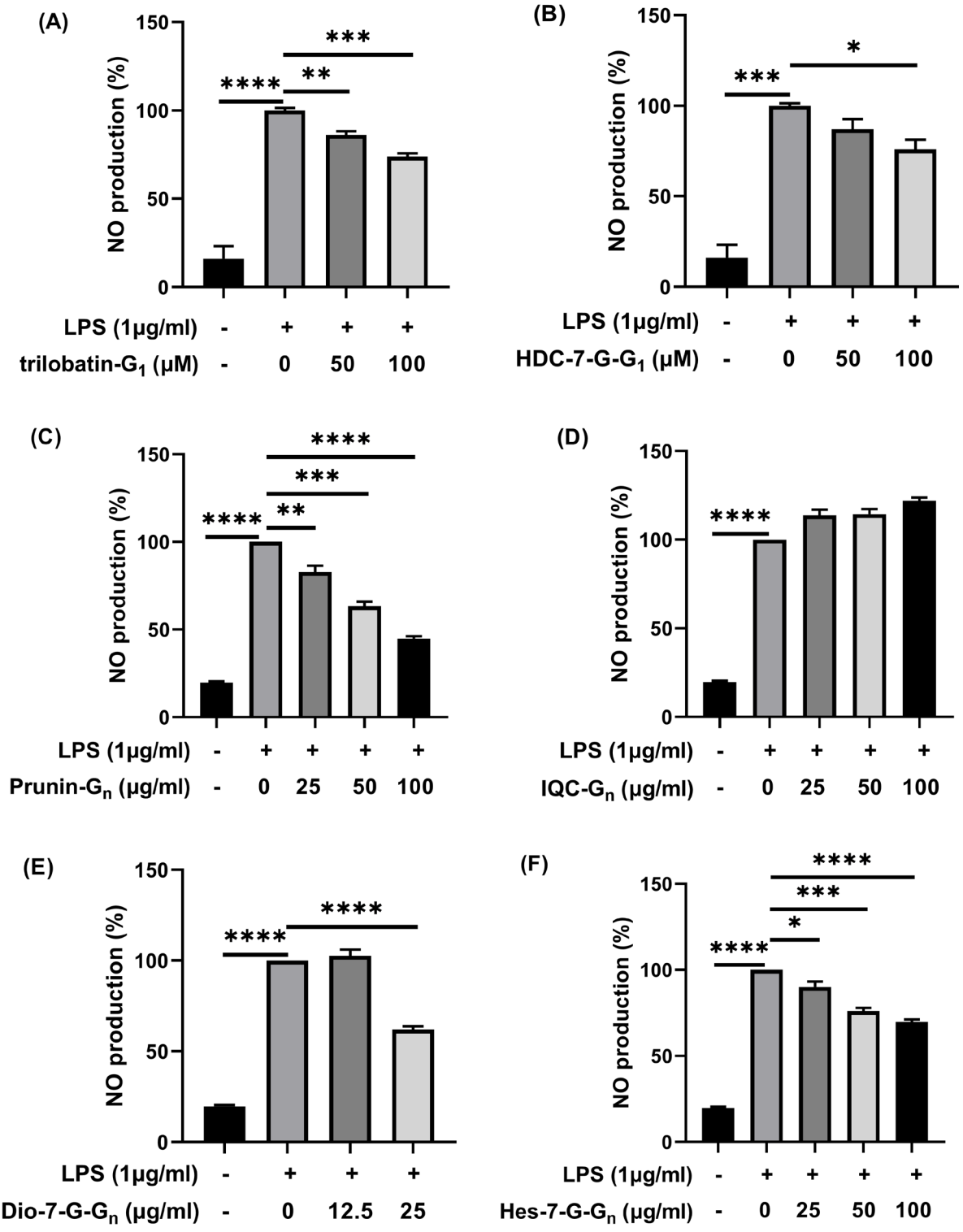
3.6 Determination of toxicity of glucosylated flavonoids to Hacat cells and PC3 cells by MTT assay

The cytotoxicity of glucosylation products **(1a-G_1 − 6a-G_1)** on human immortalized keratinocytes cells (Hacat) and human prostate cancer cells (PC3) was measured by MTT assays in the concentration range of 8-256 μM. Enzyme-linked immunosorbent assays were performed at 570 nm. The absorbance of each well was measured and the survival rate of each test compound was calculated for Hacat and PC3 cells. The

experiments were repeated three times. The results were shown in **Figure S45** in the supporting information. The results of MTT assay showed that the survival rates of both cells were above 90%, which may indicate that the compounds possess very low or even no cytotoxicity effects. Therefore, the glucosylated products may be safe for utilizing as food additives.

3.7 Evaluation of anti-inflammatory activities

The products of glucosylation were investigated for the anti-inflammatory effects against NO production induced by LPS in RAW 264.7 macrophage cells. All the tested samples at their effective anti-inflammatory concentration showed very low cytotoxicity against RAW 264.7 macrophage cells in the MTT assays (**Figure S47**). As is shown in **Figure 4**, compounds of trilobatin-G_1 (**2a-G_1**), HDC-7-G-G_1 (**4a-G_1**), prunin-G_n (**1a-G_n**), Dio-7-G_n (**5a-G_n**), and Hes-7-G_n (**3a-G_n**) were found to display potent inhibitory activities against NO production in a dose-independent manner. However, IQC-G_n (**6a-G_n**) showed no inhibitory effect. This result may indicate that the position and the number of glucosyl could be a factor affecting the anti-inflammatory effect of flavonoids. To compare the anti-inflammatory effect of the compounds, which have a closed or opened C-ring in the structure (**Scheme 1**): the compound series of analogues **1** versus that of **2**, and the series of analogues **3** versus that of **4**. We found that anti-inflammatory effect of the dihydroflavones (with a closed C-ring) was significantly higher than that of the dihydrochalcone (with an opened C-ring). Nonetheless, difference substituents in B-ring did not show obvious influence in the anti-inflammatory effect.



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438 **Figure 4.** The anti-inflammatory activity of compounds on the production of nitric oxide (NO) in
439 lipopolysaccharide (LPS)-induced RAW264.7 cells. The cells were treated with compound for 1
440 h and then stimulated with LPS (1 μg/mL) for 24 h. The inhibition of NO production was
441 calculated through OD values. (A) **2a-G$_1$**. (B) **4a-G$_1$**. (C) **1a-G$_n$**. (D) **6a-G$_n$**. (E) **5a-G$_n$**. (F)

3a-G$_n$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. All values represent the mean ± SD.

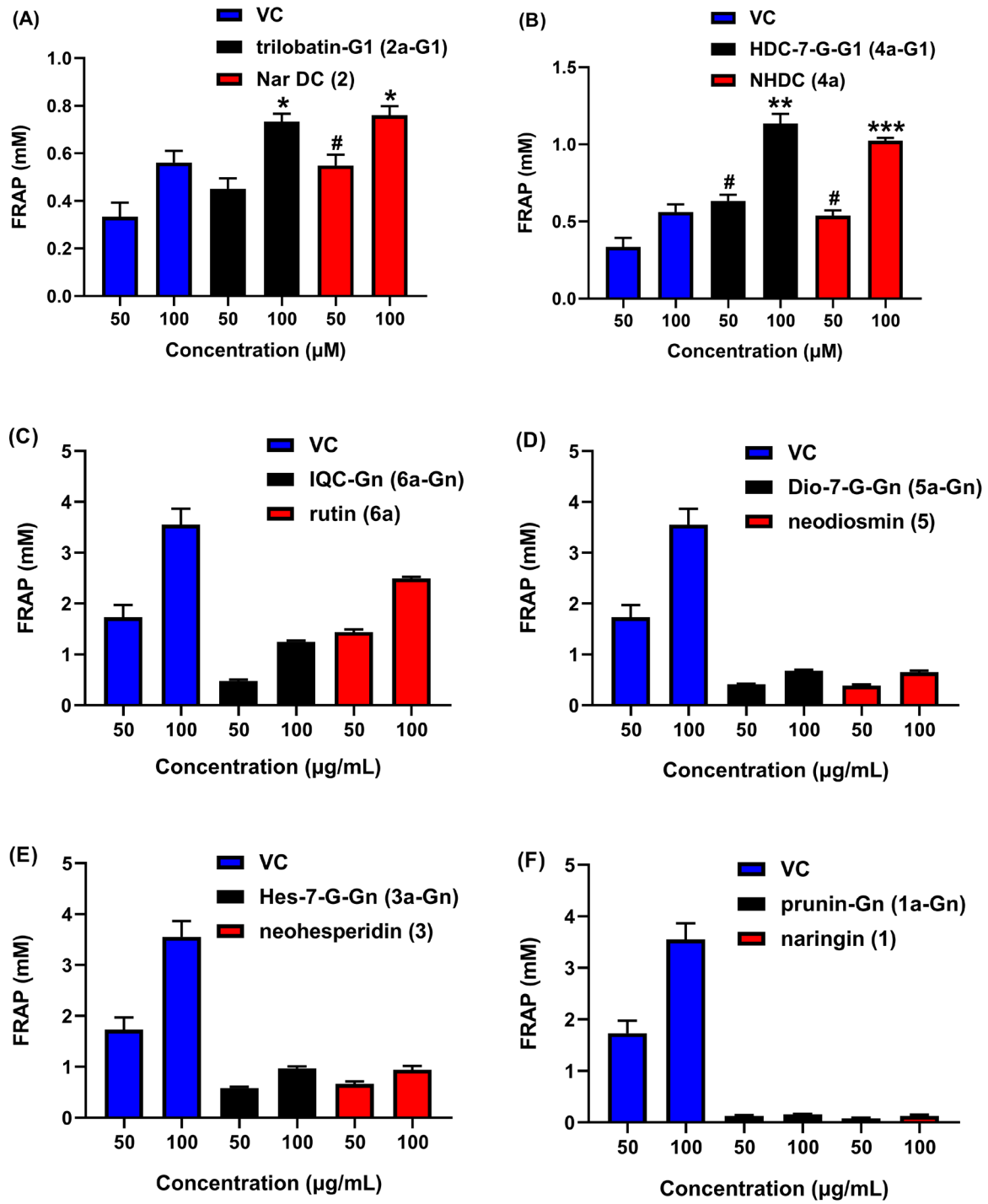

Figure 5. FRAP radical scavenging abilities of compounds. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with 100 μM or 100 μg/mL VC; $^{\#}p < 0.05$ compared with 50 μM or 50 μg/mL VC. All values represent the mean ± SD.

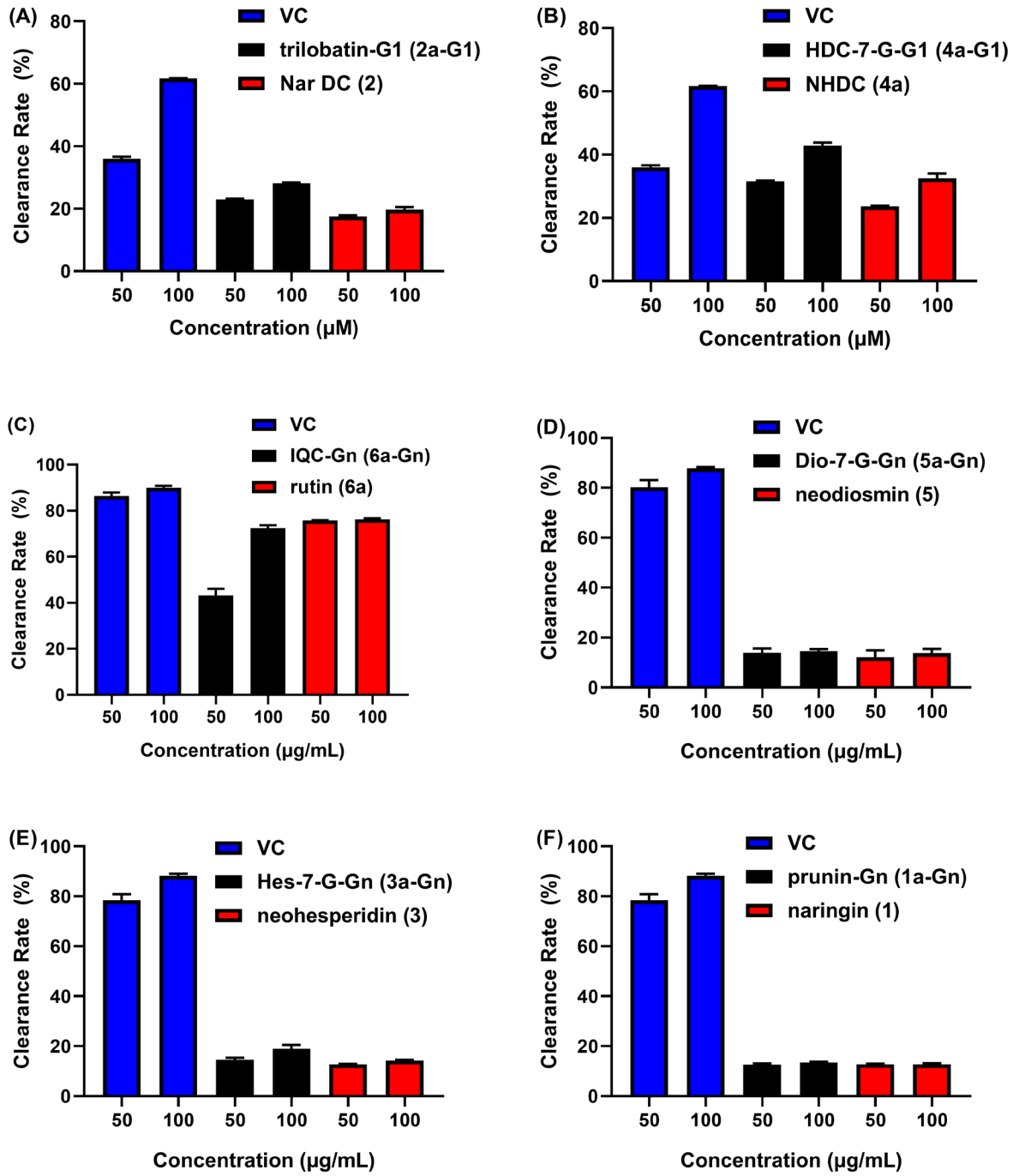

Figure 6. DPPH radical scavenging abilities of compounds. All values represent the mean ± SD.

3.8 Evaluation of antioxidant activity

FRAP is the ability that antioxidants (reductants) give electrons and reduce oxidants. The higher the FRAP content, the stronger the antioxidant capacity. Vitamin C (VC) was used as a positive control in the experiments. As shown in **Figure 5**, trilobatin-G_1 (**2a-G_1**), HDC-7-G-G_1 (**4a-G_1**), IQC-G_n (**6a-G_n**) showed excellent antioxidant effects and were found in a

dose-dependent manner. From the DPPH analysis shown in **Figure 6**, it also gave a trend similar to that was observed in the FRAP assay. From the analysis of structural-activity relationship, the antioxidation effect of dihydrochalcones (**2**, **2a-G$_1$**, **4**, **4a-G$_1$**) seems generally better than that of flavanones (**6**, **6a-G$_n$**, **5**, **5a-G$_n$**, **3**, **3a-G$_n$**, **1**, **1a-G$_n$**). In addition, by comparing the compound series of analogues **2** versus that of **4** and the series of analogues **1** versus that of **3**, it was found that different substituents of B-ring showed influence on the antioxidant effect. The antioxidant activity could be enhanced when B-ring contains a methoxy group. Furthermore, both the position and the number of glucosyl groups did not show much effect on the antioxidant activity.

3.9 Evaluation of the taste property

The taste property such as sweetness, bitterness and astringency of the compound **Nar DC (2)** (0.4 mg/mL) was measured using an INSENT Taste-Sensing System SA-402B (electronic tongue) for comparison[55]. The results of the investigation are shown in **Table S4**. In the statistical analysis of sensor precision, the values of m1 and m2 were found far less than 50, meaning that that the sensor response is correctly functioned and the test results obtained were reliable and precise.

3.9.1 Effect of concentration of compound on taste value

The results shown in **Figure 7** were prepared by converting the electrical signals obtained from the electronic tongue into a taste value. The taste value increases rapidly for the concentration of trilobatin **(2a)** from 0 to 100 μg/mL and the increment was slow down when the concentration over 100 μg/mL. Therefore, a suitable concentration of the compound for taste value evaluation was selected to be 40 μg/mL (the highlighted region in **Figure 7A** and **B**) for subsequent tests for the compounds. In addition, for trilobatin **(2a)**, taste astringency is found more dominant than sweetness and bitterness.

3.9.2 The sweetness value changes of the flavones before and after the C-ring opening.

As shown in **Figure 7C**, the sweetness value of flavanones, such as naringin **(1)**, neohesperidin **(3)**, prunin **(1a)** and Hes-7-G **(3a)**, before the C-ring opening is generally low. The sweetness values measured are less than 0.4, which indicates that these flavanones are not sweet.

However, when their C-ring was opened, the flavanones were transformed into dihydrochalcones, the sweetness values were found greatly improved and the values were higher than 1.2, which means that the taste of sweetness is enhanced remarkably.

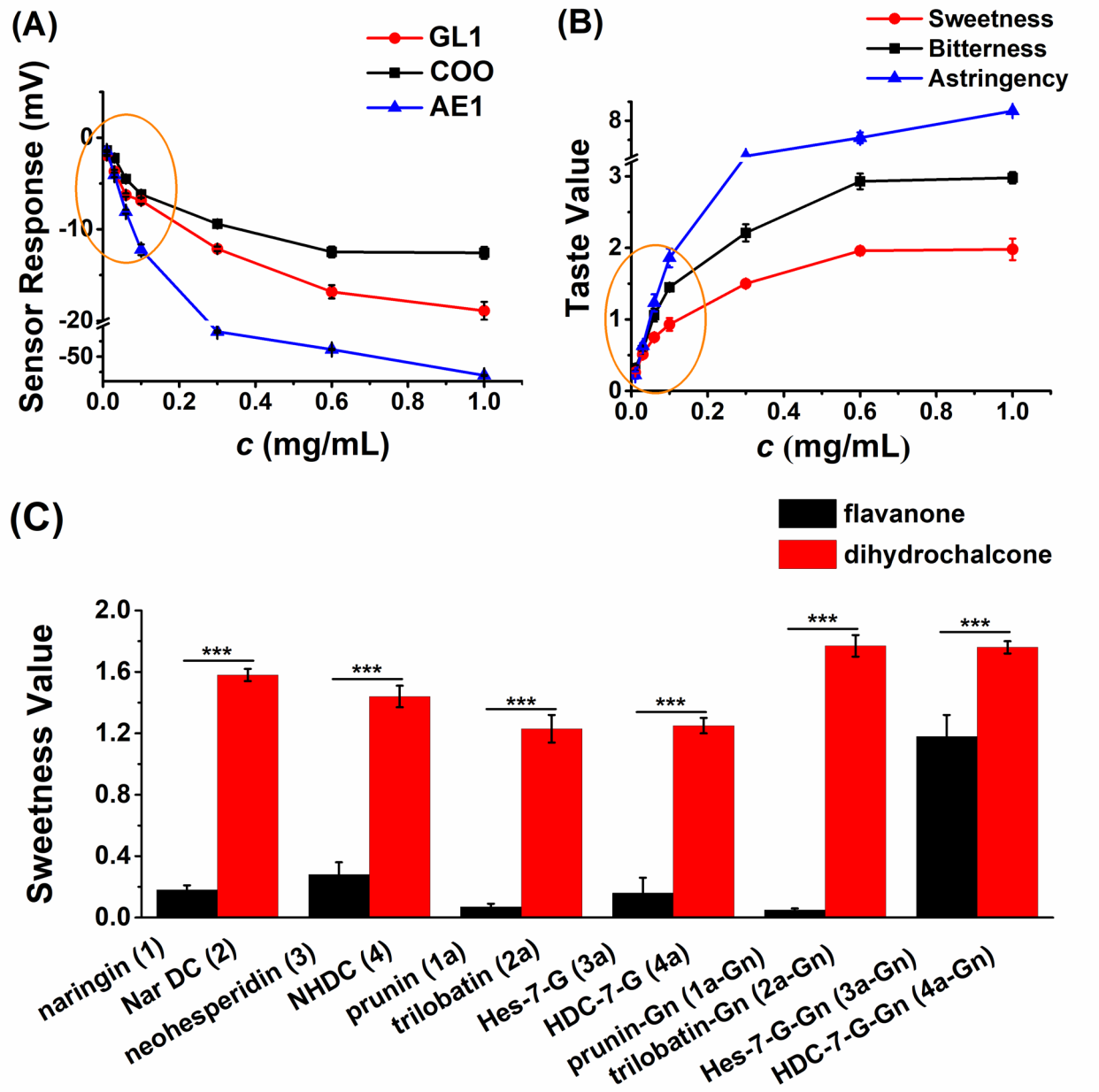

Figure 7. (A) Response of sensors (sweetness (GL1), bitterness (C00) and astringency (AE1)) for trilobatin **(2a)** in 10 mM KCl solutions with various concentrations. (B) Taste values transformed from the electrical signals obtained from (A). (C) Sweetness value comparison of flavanone and dihydrochalcone compounds. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. All values represent the mean ± SD.

3.9.3 Effect of the number of glycosyl groups in the sweetness and bitterness value

As shown in **Figure 8A**, two groups of compounds tested belong to dihydrochalcone flavonoid, which are sweet compounds. The effects of the number of glycosyl (**G**) on the sweetness value were compared because of their unexpected sweetness observed after glucosylation. In general, as the number of glycosyl groups of the compound was increased, the sweetness value was also enhanced. The sweetness value was found in the following order: monoglucoside **(1a－6a)** < diglycoside **(1－6)** < polyglucoside **(1a-G_n－6a-G_n)**. Moreover, for the bitter compounds of flavone and flavanone shown in **Figure 8B**, the bitterness value of monoglucoside **(1a－6a)** (after the removal of rhamnosyl group) was found higher than the diglycoside **(1－6)**. When adding poly-glucosyl groups (G_n) to the monoglucosides **(1a－6a)**, the bitterness value was also increased significantly. This may imply that the addition of glucosyl groups enhance the bitter taste of the compound of flavone and flavanone. In general, the order of bitterness value of flavone and flavanone compounds was found as follows: diglycoside **(1－6)** < monoglucoside **(1a－6a)** < polyglucoside **(1a-G_n－6a-G_n)**.

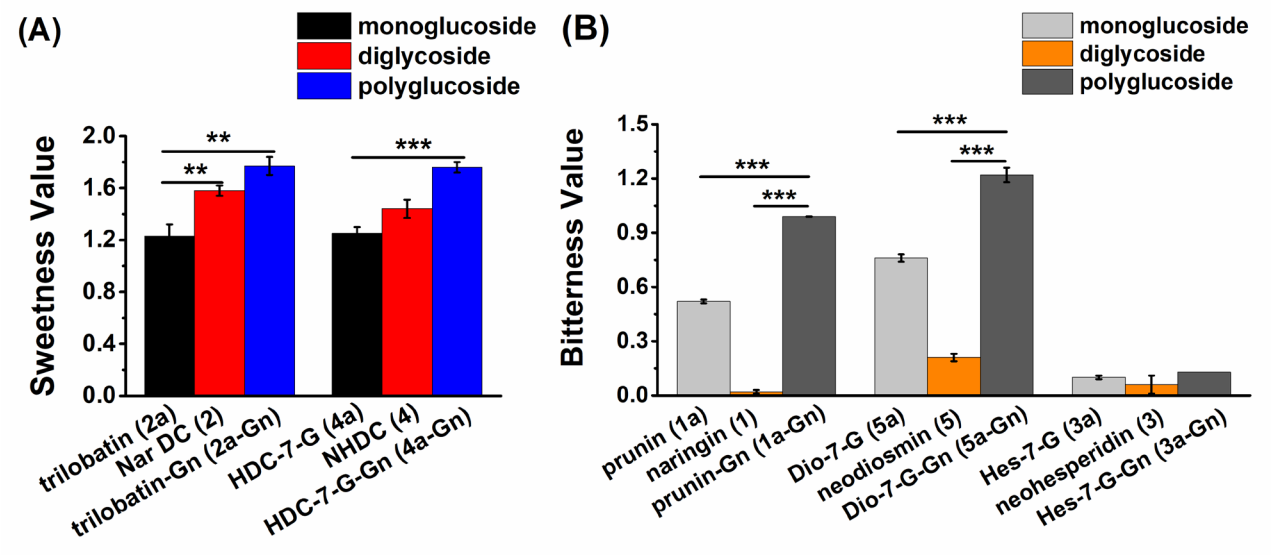

Figure 8. Taste values comparison of flavonoid compounds before and after enzyme glucosylated: (A) dihydrochalcone compounds; (B) flavone and flavanone compounds. *P < 0.05, **P < 0.01, and ***P < 0.001. All values represent the mean ± SD.

Conclusion

In conclusion, we demonstrated an efficient enzymatic method to produce twelve new glucosylated compounds from some flavonoids that are naturally available from citrus peel extracts. It was found that the removal of rhamnosyl group of flavonoid compounds enhances slightly the water solubility while the glucosylation is able to increase water solubility remarkably. The results support that the introduction of glucosyl groups improve water solubility of flavonoid compounds for food formulation and process. In addition, the log $P_{o/w}$ value of the glucosylated compounds was reduced slightly and was within a suitable range ($-1 < \log P_{o/w} < 2$). The modified products also exhibited good anti-inflammatory and antioxidant effects. Furthermore, the taste evaluation study showed that the structure and number of glycosides of citrus flavonoids have significant influence on their taste perception. Taken together, our study optimized the enzymatic glucosylation reaction process for structure modification of citrus flavonoids and thus altering their biological activity and taste property. The results may be able to provide a new approach for flavonoid-based product development and application in the fields of healthy foods and pharmaceuticals.

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Declaration of competing interest

The authors declare no conflict of interest.

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