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Research article

Synthesis and evaluation of antioxidant and β-glucuronidase inhibitory activity of hesperidin glycosides

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Abstract

Cyclodextrin glycosyltransferase (CGTase) catalyzes an intermolecular transglycosylation reaction to produce functional oligosaccharides or glycosides. Such products are used in the food and drug industry to improve bioavailability. Previous work purified recombinant CGTase and identified hesperidin as a flavonoid acceptor. The current study optimized the conditions for recombinant CGTase to convert β-cyclodextrin (β-CD) and hesperidin to three major products identified using mass spectrometry: hesperidin glucoside (HG_1), hesperidin maltoside (HG_2) and hesperidin maltotrioside (HG_3), whose molecular weights were 754 Da, 916 Da and 1,078 Da, respectively. They were hesperidin glycosides with 1, 2 and 3 monosaccharide units. The solubility and free-radical scavenging properties of HG_1 and HG_2 were increased compared to original hesperidin but they had lower anti-inflammatory activity by inhibiting less β-glucuronidase and all properties were dependent on the number of glucose or maltose molecules attached to the -OH groups of hesperidin. The results suggested hesperidin glycosides could replace hesperidin in food and drugs and more work is needed to see whether their antioxidant properties can be exploited.

Introduction

Cyclodextrin glycosyltransferase (CGTase, E.C.2.4.1.19) catalyzes four related reactions: cyclization (intramolecular transglycosylation), coupling, disproportionation and hydrolysis (intermolecular transglycosylation) (Kitahata et al., 1979). The main metabolic products from its intermolecular transglycosylation are functional oligosaccharides and glycosides that are useful in several industries (Kitahata et al., 1979). Functional oligosaccharides and glycosides can be synthesized in vitro by using various donor and acceptor substrates (Bousquet et al., 1998; Svasti et al., 2003). The demand for glycoside and oligosaccharide products in food, drinks and cosmetics has increased because they usually have better characteristics (such as solubility, stability and bioactivity) than their parent compounds. For example, 3-*O*-α-maltosyl-L-ascorbate, synthesized from α-maltosyl fluoride and L-ascorbic acid and catalyzed by mutated CGTase, has high stability under oxidative conditions (Ahn et al., 2015). Reports are available on intermolecular transglycosylation to produce useful glycosides by the action of CGTase (Charoensapyanan et al., 2017; Chotipanang et al., 2011; Wongsangwattana et al., 2010).

Flavonoids have also been used as substrates for the production of functional glycosides (Kometani et al., 1996, 1994; Aramsangtienchai et al., 2011). Flavonoids encompass a wide range of plant phenolic compounds and there are currently more than 5,000 different flavonoids. They are divided in to six classes: flavones (such as flavone, apigenin and luteolin), flavonols (such as quercetin, kaempferol, myricetin and fisetin), flavanones (such as hesperetin, hesperidin and naringenin), flavanonol (such as taxifolin), isoflavones (such as genistein) and

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flavan-3-ols (such as catechin and epicatechin). Chemically, flavonoids consist of two benzene rings A and B linked via a heterocyclic pyran ring C. Different classes of flavonoids have differences in the level of oxidation and patterns of replacement of the C ring while within the same class there are differences in the pattern of replacement of the A and B rings (Wang et al., 2018). Flavonoids share a basic skeleton C6-C3-C6 phenyl-benzopyran structure and these can be found in two different forms: the free form (aglycones) and the bonded form with the attachment of glycosides (de Villiers et al., 2016).

Flavonoids are widely used in pure and mixed forms in various drugs and food. Many biological activities of flavonoids have been described, including inhibitory effects on mammalian enzymes (Proença et al., 2017), antiviral activity (Zakaryan et al., 2017) and antioxidant (Procházková et al., 2011), anti-inflammatory (Middleton Jr and Kandaswami, 1992) and anti-carcinogenic (Liu et al., 2010) activities. Although they have such useful properties, their use is limited because of their low water solubility.

Glycosylation of a flavonoid affords structural complexity and diversity (Yang et al., 2018) and various flavonoids have been used as acceptor substrates in CGTase transglycosylation reactions such as naringin (Kometani et al., 1996), hesperidin (Kometani et al., 1994) and epicatechin (Aramsangtienchai et al., 2011). It is known that most flavonoids are more water soluble when they are transglycosylated with saccharides (Kometani et al., 1996) and this should improve their bioavailability when used in food and drugs.

Previous study on acceptor specificity (Chaisin et al., 2018) found among several flavonoids, that hesperidin was the best acceptor for this CGTase. Hesperidin is a natural flavanone glycoside, comprising an aglycone, hesperetin and an attached disaccharide, rutinose (Fig. 1). Hesperidin is abundant in citrus fruits like mandarins, oranges and lemons (Pandey and Rizvi, 2009). There is evidence that hesperidin has antioxidant (Parhiz et al., 2015), antibacterial (Corciova et al., 2015), anti-inflammatory and anti-proliferative (Cincin et al., 2015) and anti-cancer (Cincin et al., 2018; Xia et al., 2018; Pandey et al., 2019) activities. Overall, hesperidin seems to be an inexpensive, less toxic and environment-friendly flavonoid, having useful potentially applications in biotechnological and biomedical industries. Although it has a sugar molecule in its structure, hesperidin is still not easily soluble in water. So, the current study investigated the production of hesperidin glycosides with the aim of increasing their solubility and hesperidin bioactivity using the recombinant CGTase (Rimphanitchayakit et al., 2005; Kaulpiboon et al., 2010).

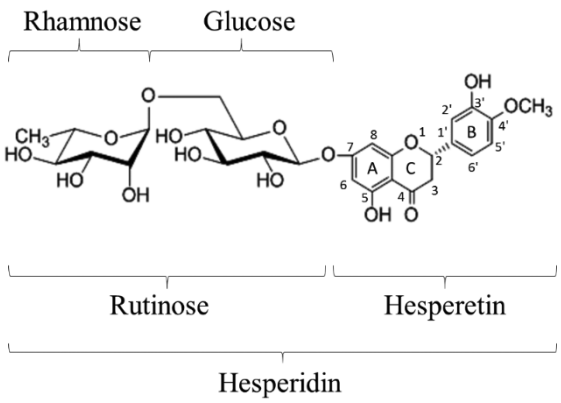

Fig. 1 Structure of hesperidin showing core structure of hesperitin (flavonoid ring) and rutinose (glucose + rhamnose); sourced: Sammani et al. (2017)

The current study synthesized and characterized new hesperidin glycosides. In addition, the solubility in water and in vitro antioxidant and anti-inflammation activities of synthesized hesperidin glycosides were also evaluated.

Materials and Methods

Chemicals

The pBC recombinant cell (a pET-19b based plasmid containing the CGTase gene with a signal peptide sequence from *Bacillus circulans* A11 in *E. coli* strain BL21 (DE3) host cell), was obtained from the Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand (Kaulpiboon et al., 2010). Crude CGTase from the pBC recombinant cell culture was prepared followed the method of Chaisin et al. (2018). Hesperidin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals used were of analytical grade. Unless otherwise specified, laboratory chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

Detection of hesperidin glycoside products

Optimization of transglycosylation reaction

The optimal conditions for the synthesis of hesperidin glycosides were considered in terms of obtaining the highest percentage yield of the transglycosylated products, which were analyzed using thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The effects were investigated of varying dimethyl sulfoxide (DMSO) concentrations as a resuspension solution for hesperidin (20–100%), the pH (3.0–11.0), enzyme concentration (20–200 U/mL), β-CD concentration (0.5–2.5% weight per volume (w/v)), hesperidin concentration (0.25–2.5% (w/v)), temperature (30–80°C) and incubation time (0.5–72 hr). The synthesis of hesperidin glycosides was then performed under the optimized conditions obtained above. Thereafter, hesperidin glycosides were separated using HPLC. Each glycoside-containing peak was collected for further characterization.

Thin layer chromatography analysis

Hesperidin glycoside products were analyzed on TLC silica gel 60 F_{254} 20 cm in height (Merck; Darmstadt Germany). The mobile phase composed of ethyl acetate:acetic acid:water (3:1:1, volume per volume (v/v)) was used for running a TLC plate (Charoensapyanan et al., 2017). After running once, spots were detected by spraying with 1:2 (v/v) ratio of sulfuric acid: methanol followed by heating at 120°C for 20 min. The intensity of the synthesized product spots was quantified using a scanning densitometer. A glucose spot (5 µg) was set as the standard value.

High performance liquid chromatography analysis

The products were analyzed using HPLC (Agilent Technologies 1260; Waldbronn, Germany) using a 5 µm reverse phase C_{18} column (4.6 × 250 mm; Phenomenex; Torrance, CA, USA) and detected using an ultraviolet (UV) detector at 280 nm. The reaction mixture of glycoside product was filtered through a nylon membrane disc filter (width 0.45 µm) before injection and elution with acetonitrile:water (20:80, v/v) using a flow rate of 0.5 mL/min at 40°C (Kometani et al., 1996). Product yield as a percentage was determined as (peak area of product / peak area of acceptor at t_0) × 100, where t_0 is the commenced time.

Mass spectrometry of hesperidin glycosides

The hesperidin glycoside products were dissolved in 50% (v/v) methanol and put into the mass spectrometer. The electrospray ionization-time of flight mass spectrometry (ESI-TOF MS) profile was recorded on a microTOF (Bruker; Ettlingen, Germany) at the Service Unit of the National Center for Genetic Engineering and Biotechnology (BIOTECH), Thailand. The compounds were ionized using electrospray ionization in the sodium positive-ion mode using a capillary voltage of 5,000 V. A flow of 4 L/min of nitrogen gas at 150°C was established to nebulize the analytic solution to droplets using a nebulizer pressure set at 1 bar. A mass spectrometer (Bruker; Ettlingen, Germany) was used to determine the molecular mass of chemical compounds by separating ionic molecules according to their mass-to-charge ratio (m/z). The molecular mass was calculated as (molecular mass + number of protons) / charge = m/z.

Determination of the properties of hesperidin glycosides

Solubility in water

Excess amounts of hesperidin glycosides (HG_s:HG_1 and HG_2) were mixed with 200 µL of water, vortexed for 5 min at room temperature to disperse into solution; then, each sample was incubated at 30°C for 15 min to ensure saturation. Hesperidin glycosides were then filtered through a 0.45 µm membrane to remove insoluble particles and subjected to quantitative reverse phase C_{18} HPLC analysis to determine the concentration of soluble hesperidin glycoside (Aramsangtienchai et al., 2011). The soluble part of the sample was analyzed using HPLC and the solubility of each hesperidin glycoside was compared to that of hesperidin using a standard hesperidin solubility curve.

Anti-oxidant agent

The antioxidant activity of hesperidin and hesperidin glycosides (HG_1 and HG_2) was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging reaction (Abe et al., 2000). Hesperidin and its glycosides were dissolved or diluted in the required volume of ethanol to yield the final hesperidin glycoside concentration in the range 0–2,400 µM. DPPH was then added to a final concentration of 100 µM. After 10 min in the dark, the absorbance of the mixture was measured at 517 nm. The DPPH radical scavenging activity was evaluated by analyzing the percentage decrease in the absorbance of the sample, compared to ethanol as a control. The IC_{50} value designates the inhibitory concentration at which the absorbance was reduced by 50%.

β-Glucuronidase inhibition assay

For this anti-inflammatory assay, 0.1 mL of 2.5 mM *p*-nitrophenyl-β-D-glucopyranosiduronic acid in 0.1 M acetate buffer (pH 7.4) was incubated with 1 mg of hesperidin or its glycoside derivatives for 5 min followed by the addition of 0.1 mL of β-glucuronidase. The reaction mixture was then incubated for 30 min followed by the addition of 2 mL of 0.5 N NaOH to halt the reaction. The amount of reaction product formed was measured at 410 nm and the β-glucuronidase inhibitory (anti-inflammatory) activity was calculated using Equation 1, with 1 mM of salicylic acid solution used as a reference for comparison (Nia et al., 2003):

$$\text{Anti-inflammatory activity (\%)} = [1 - (OD_{410} \text{ of sample} / OD_{410} \text{ of control})] \times 100 \quad (1)$$

Statistical analyses

All data were expressed as the mean ± SD from at least three separate experiments and the differences were calculated using an unpaired Student's t test with the GraphPad Prism version 5.0 software (GraphPad Software Inc, La Jolla, CA, USA) and *p*-values < 0.05 were considered statistically significant.

Results and Discussion

Transglycosylation to hesperidin and optimization of transglycosylation reaction

Previous study found that the appropriate donor and acceptor for the synthesis of glycoside products were β-CD and hesperidin, respectively, with at least three products being observed using TLC and five using HPLC (Chaisin et al., 2018). In the current study, to determine the appropriate conditions for the production of hesperidin glycosides, various parameters involved in the reaction were optimized: solvent concentrations, donor concentrations, acceptor concentrations, enzyme concentration, pH, incubation time and temperature. The optimal conditions for the production of hesperidin glycosides using recombinant CGTase from *B. circulans* A11 were incubation at 60°C for 6 hr and 1.5% (w/v) β-CD with 2% (w/v) hesperidin in 80% (v/v) DMSO using 200 U/mL CGTase in acetate buffer, pH 5.0 (Fig. 2). After optimization, the total yield of hesperidin glycosides increased slightly compared with the yield obtained before optimization (Table 1). It was found that the peaks with retention time values of 17.60, 14.58, and 11.56 min, corresponded to HG_1, HG_2, and HG_3, respectively (Fig. 3).

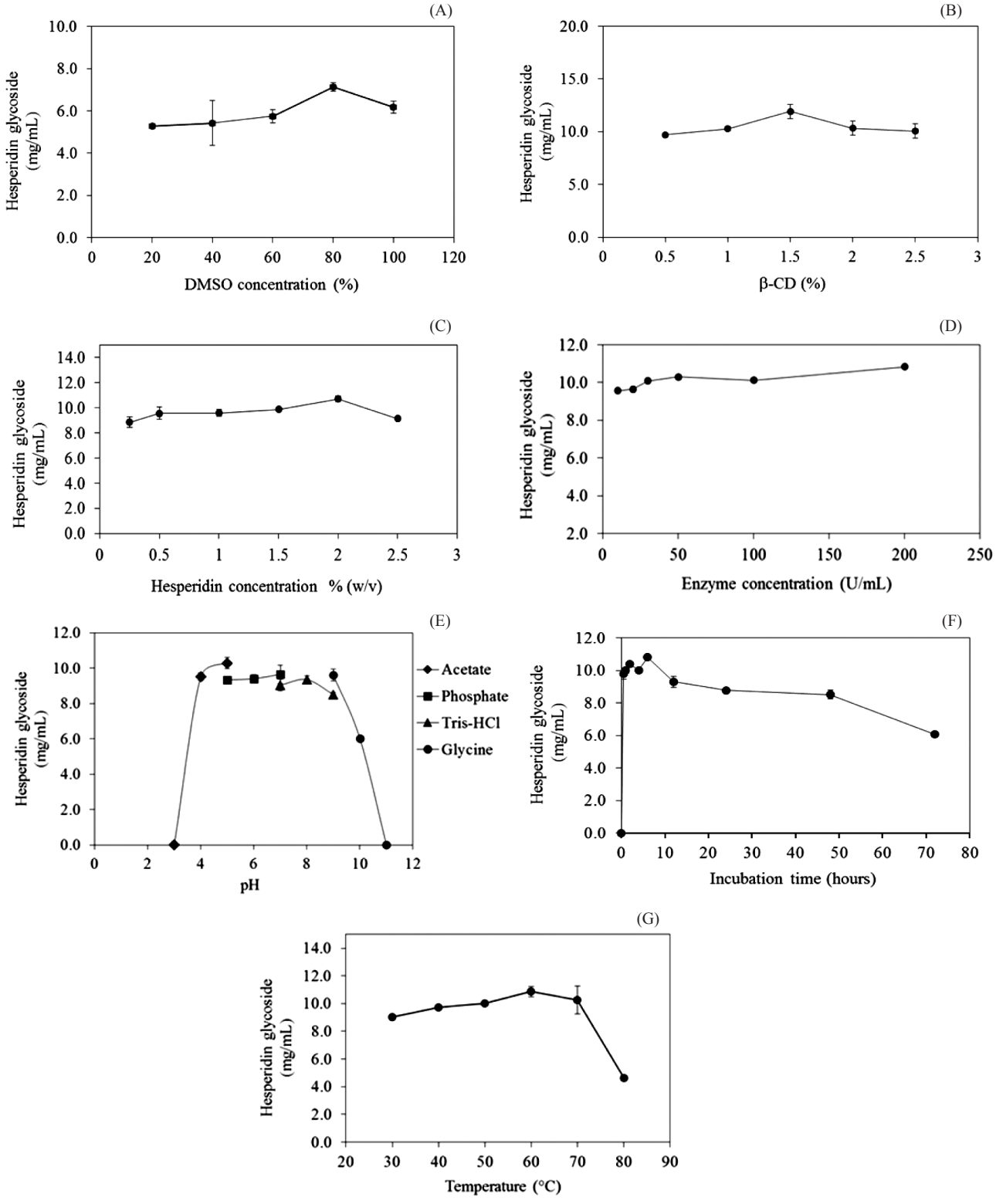

Fig. 2 Hesperidin glycoside synthesis as affected by: (A) dimethyl sulfoxide (DMSO) concentration; (B) β-cyclodextrin (β-CD) concentration (donor substrate); (C) hesperidin concentration (acceptor substrate); (D) enzyme CGTase concentration; (E) pH; (F) temperature; (G) incubation time, where w/v = weight per volume and error bars indicate ±SD

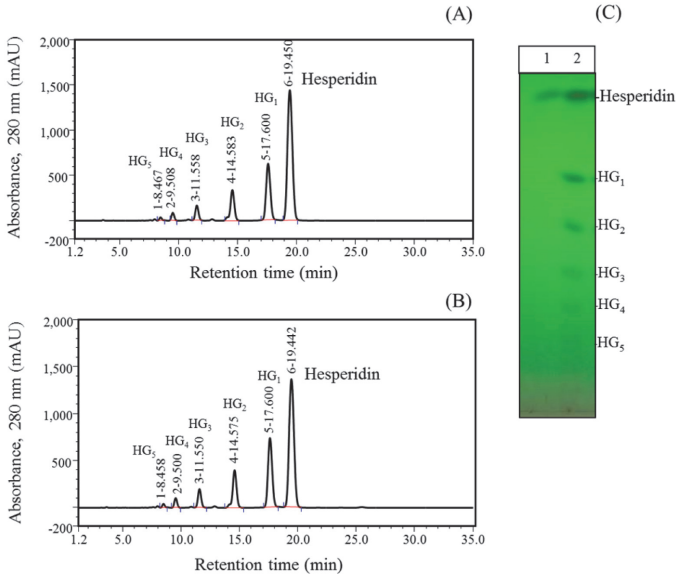

Fig. 3 Detection of hesperidin glycoside products using high performance liquid chromatography profile of transglycosylation reaction catalyzed using CGTase: (A) before optimization; (B) after optimization; (C) thin layer chromatogram of the transglycosylation products of hesperidin detected under UV 254 nm under optimal conditions (lane 1: control reaction at 0 hr incubation; lane 2: HG products at 6 hr incubation)

Table 1 Yields of hesperidin glycoside products before and after optimization

Reaction	Product yield (%)		Total yield (%)
	HG_1 (R_t =17.60 min)	HG_2 (R_t =14.58 min)	
Before optimization	22.08±1.05*	11.13±0.54*	33.21±1.59*
After optimization	24.80±1.13*	12.76±0.86*	37.56±1.99*

R_t = retention time.
values shown as mean ±SD.
*significantly differ between before and after optimization ($p < 0.05$)

Mass spectrometry of hesperidin glycosides

Mass spectrometry (MS) was used to determine the mass-to-charge ratio of hesperidin glycoside products. A mass spectrum is a plot of the ion signal and spectra are used to determine the elemental or isotopic signature of a sample, the mass of particles and of molecules, and to elucidate the chemical structures of molecules such as peptides and other chemical compounds. The molecular weights (MWt) of hesperidin and its products were determined using ESI-TOF mass spectrometry. The MWt of standard hesperidin was $[M+Na]^+$ at an m/z of 633.18 (Fig. 4A, Table 2) and this corresponded to the exact size of hesperidin. The MWt of product 1 (HG_1) was $[M+Na]^+$ at m/z of 795.23 (Fig. 4B, Table 2) was equivalent to the molecular weight of a single glucose unit attached to hesperidin. The MWts of products 2 (HG_2) and 3 (HG_3) were also $[M+Na]^+$ at m/z of 957.29 (Fig. 4C) and 1119.34 (Fig. 4D), respectively, indicating two and three glucose units, respectively, that were attached to hesperidin. These results confirmed the successful transglycosylation of glucose from β-CD by the recombinant CGTase to produce hesperidin and three hesperidin glycoside products which contained hesperidin glucoside (HG_1), hesperidin maltoside (HG_2) and hesperidin maltotrioside (HG_3).

Table 2 Calculated and determined molecular weights of hesperidin and hesperidin glycosides

Product	Mass number (m/z)	
	Calculated[a] $[M+Na]^+$	Determined[b] $[M+Na]^+$
Hesperidin (H)	633.56±0.00	633.18±0.07
Hesperidin glucoside (HG_1)	795.56±0.00	795.23±0.13
Hesperidin maltoside (HG_2)	957.56±0.00	957.29±0.19
Hesperidin maltotrioside (HG_3)	1119.56±0.00	1119.34±0.16

values shown as mean ±SD.
[a] mass numbers were calculated from glucose (G_1) = 180.16, maltose (G_1-G_1) = 342.30, maltotriose (G_1-G_1-G_1) = 504.44, Na = 22.98; mass number of hesperidin = 610.56, H_2O = 18.02.
[b] $[M+Na]^+$ were determined using electrospray ionization-time of flight mass spectrometry.

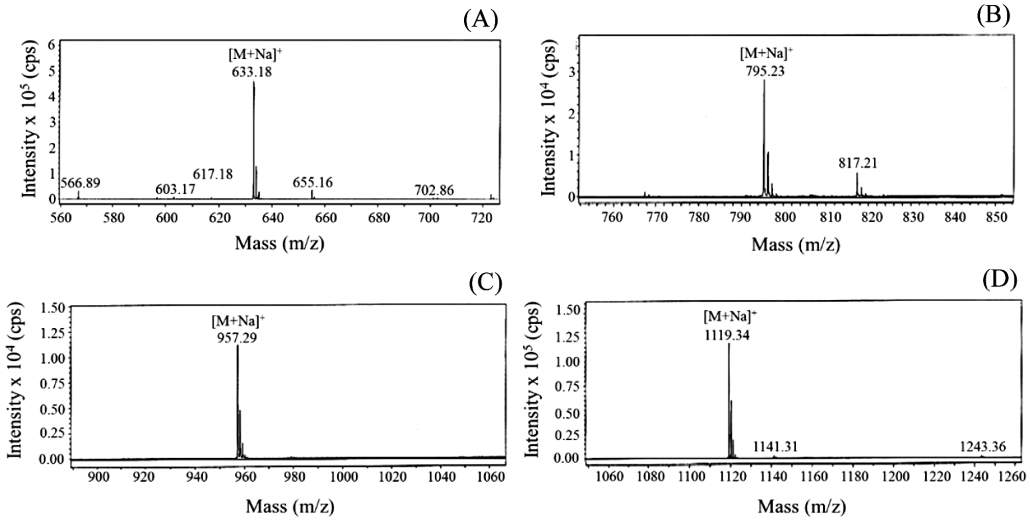

Fig. 4 Electrospray ionization-time of flight mass spectrometry mass spectra of: (A) hesperidin; (B) hesperidin glucoside (HG_1); (C) hesperidin maltoside (HG_2); (D) hesperidin maltotrioside (HG_3)

Determination of hesperidin glycoside properties

Solubility

The water solubility of hesperidin and HG_1 and HG_2 were calculated using a standard hesperidin solubility (in DMSO) curve. Hesperidin itself was water insoluble but the solubility of HG_1 and HG_2 was 533.5 and 582.5 mg/mL, respectively, with HG_1 being less soluble than HG_2. This result suggested that the addition of glucose units led to increased numbers of hydroxyl groups in hesperidin molecule; consequently, HG_2 has more hydroxyl groups with a higher hydrophilicity than HG_1. In addition, Kometani et al. (1996) reported that the solubility greatly improved when adding more glucose molecules to the flavonoid structure and showed that the solubility of naringin monoglucoside increased up to 1,000 times higher than that of naringin. They also synthesized 3^G-α-D-glucopyranosyl neohesperidin using CGTase and found that its water solubility was 1,500-fold greater and 10-fold less bitter than its parent, neohesperidin.

The greater degree of water solubility allows for greater absorption from the gastrointestinal tract. Flavonoid glycoside had better absorption than flavonoid and the addition of glucose to flavonoid aglycone improved its bioavailability due to the involvement of the glucose transporter (Chen et al., 2014; Yang et al., 2018). Thus, adding glucose to the original structures of flavonoid or hesperidin increased both their water solubility and bioavailability.

Anti-oxidant agent

The DPPH radical scavenging assay is commonly used to measure antioxidant activity (Abe et al., 2000). In the current study, HG_1 and HG_2 inhibited DPPH radicals in a dose-dependent manner. The IC_{50} value is the effective concentration of an antioxidant to scavenge 50% of the DPPH radicals; a lower IC_{50} value indicates greater antioxidant activity (greater DPPH scavenging). The IC_{50} values of hesperidin, HG_1 and HG_2 were 1192.39, 1015.09 and 564.81 μM (Fig. 5), respectively, compared to the standard trolox (IC_{50} = 4.51 μM). Thus, HG_2, HG_1 and hesperidin (H) had less scavenging capacity than trolox but the trend of increased scavenging capability of HG_2 vs. > HG_1 vs. > H suggested that adding more glucose to hesperidin resulted in increased antioxidant activity. The anti-oxidant activity of flavonoid may also be directly correlated with the number of hydroxyl groups and the degree of *O*-methylation and glycosylation. Moreover, the number of glycosyl residues, their position and the structure of the saccharide are also significant determinants (Wang et al., 2018) as: 1) an *ortho*-dihydroxy structure in the B ring; 2) 2, 3-double bond in conjugation with a 4-oxo function in the C ring; and 3) hydroxyl groups at positions 3 and 5 provide hydrogen bonding to the oxo group (Procházková et al., 2011). Hesperidin has a hydroxyl group at the 3' of ring B (Fig. 1), which may be responsible for the capacity of hesperidin to scavenge the hydroxyl radicals (Wilmsen et al., 2005). In addition, (van Acker et al. (1996) reported that the ring B C-4' methyl substitution of hesperidin can activate the ring B C-3' hydroxyl group, making hesperidin a better scavenger of free radicals. Hesperidin exerts its major antioxidant properties via two ways: direct radical scavenging and augmenting cellular antioxidant defense (Procházková et al., 2011). A number of studies have shown that hesperidin neutralized reactive oxygen species, including superoxide anions, hydroxyl radicals, peroxynitrite (Kim et al., 2004) and nitric oxide radicals (Garg et al., 2001; Wilmsen et al., 2005) and that the mechanism is the induction of heme oxygenase-1 (HO-1) expression via the ERK/Nrf 2 signaling pathway (Elavarasan et al., 2012; Martínez et al., 2009; Parhiz et al., 2015). The anti-oxidant activity of hesperidin plays an important role in their protection of DNA, proteins and tissues against damage that is induced by intrinsic (such as oncogenes) and extrinsic (such as radiation, inflammation and toxins) factors (Parhiz et al., 2015).

β-Glucuronidase inhibition assay

Para-nitrophenyl β-D-glucosiduronic acid is proposed as a substrate for the assay of the hydrolytic activity of β-glucuronidase; β-glucuronidase itself is related to inflammation. The β-glucuronidase inhibitory (anti-inflammatory) activities of hesperidin, HG_1 and HG_2 were 51.34, 23.79 and 16.44%, respectively (Fig. 6), suggesting that an increased number of glucose molecules in hesperidin decreased the β-glucuronidase inhibitory activity. This was probably due to glucose-induced β-glucuronidase activity rather than substrate inhibition in

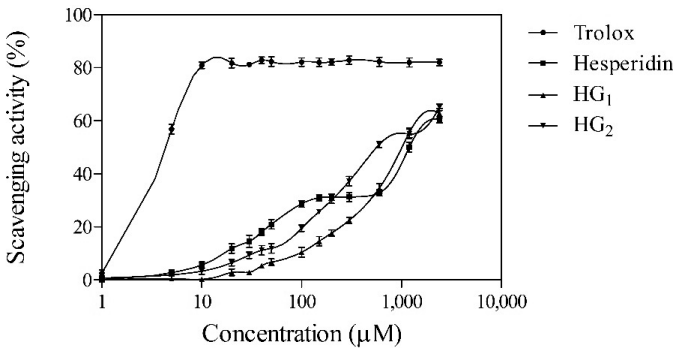

Fig. 5 DPPH radical scavenging activity of trolox, hesperidin, hesperidin glucoside (HG_1), hesperidin maltoside (HG_2), where data shown are the mean of six replicates and error bars indicate ±SD

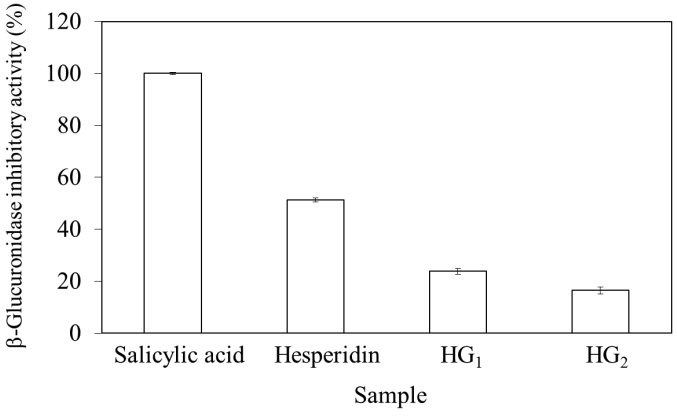

Fig. 6 β-Glucuronidase inhibitory (anti-inflammatory) activity of salicylic acid, hesperidin, hesperidin glucoside (HG_1), hesperidin maltoside (HG_2), where data shown are the mean of six replicates and error bars indicate ±SD

the assay. Isoda et al. (2014) suggested that glycosides with lower lipophilicity have lower anti-inflammatory activity because the lower hydrophobicity and sterical hindrance decreased membrane permeability in the cell culture. However, Rudeekulthamrong and Kaulpiboon (2016) investigated the anti-inflammatory properties of salicin glycosides, using the β-glucuronidase inhibition assay, and showed that salicin with a long chain carbohydrate (Sa-G_3) was more effective at inhibiting β-glucuronidase than salicin with a short chain carbohydrate (Sa-G_1).

Hesperidin inhibits the function of pro-inflammatory mediators such as nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which play roles in the production of nitric oxide (NO) and prostaglandins (PGs), respectively (Kang et al., 2011). Various in vitro and in vivo studies of different designs have been conducted to evaluate the anti-inflammatory effects of hesperidin and its metabolites (Parhiz et al., 2015). Although long chained flavonoid glycosides were less effective at inhibiting β-glucuronidase, they had greater solubility and antioxidant properties.

In conclusion, using recombinant CGTase the current study extracted and characterized at least two hesperidin glycosides which had greater water solubility and anti-oxidant properties but weaker β-glucuronidase inhibition compared to hesperidin. These results suggested promising results from using hesperidin glycosides to improve the bioavailability of foods and drugs. More work should be done using nuclear magnetic resonance to determine the specific position of the glucose moiety linkage in hesperidin glycosides. Moreover, there is scope for studying the stability and anti-bacterial and anti-proliferative properties of hesperidin glycosides. While the current work was confined to in vitro experimentation, it is now time to progress to animal models.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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